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Study of bacteria involved in *Acacia longifolia* nodulation: influence of fire on symbiosis establishment

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"Ouve aquilo que te digo, já li num artigo"
que é uma referência transformada em hipóteses, pensando em Lavoisier

E para quem *"Calça as botas e vai e veste a bata e vem":*
A curiosidade é mandatória,

E aqui fica o meu primeiro *"Era uma vez"*

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A todos e a cada um, aquela palavra de 8 letras que não chega e um abraço nosso!

Abstract

Acacia longifolia is an invasive leguminous species that can cause a considerable damage in the ecosystems. This species, native from coastal dunes of southern Australia, was brought to Portugal for dune stabilization, preserve sand erosion and ornamental purposes, competing with native plants for natural resources and changing the soil characteristics. The expansion of this species, in the last decades, caused an environmental impact, in particularly in the Portuguese coastal dunes. Several factors potentiate its expansion, namely fires that occur frequently and promote seed germination. Furthermore, high colonization success of *A. longifolia* is related to its association with nitrogen-fixing bacteria, that develop inside root nodules, where fixation occurs. These bacteria, belonging mainly to the Rhizobiaceae family are determinant in plants' growth. Out study aimed to understand (1) which bacteria (genus/species) are present in the symbiosis; (2) if fire will alter the bacterial community established in the nodules; and (3) nodule morphological characterization.

Nodules from 20-60 cm long young plants of *A. longifolia* were collected in the field in unburnt zone and burnt zone (one-year after fire). For bacterial isolation, disinfection protocol was applied in nodules and a total of 152 bacterial isolates were obtained in YMA growth medium. DNA was extracted and genomically fingerprinted by PCR amplification with M13 and GTG-5 universal primers. Assessment of diversity was based on dendrograms with Pearson similarity coefficient and UPGMA. The amplification for rRNA 16S gene was performed to identify the main bacterial genera involved in symbiosis. Next Generation Sequencing was achieved to complement bacterial diversity data for the first time in nodules. Alongside, soil characterization and isotopic analysis (N and C) were done to understand outside and inside nodule environments, respectively.

A total of 152 isolates were obtained, 92 from unburnt zone and 60 from burnt zone. A higher bacterial diversity was observed in unburnt zone and high specific symbionts were found in burnt zone in what concerns nitrogen fixation. *Bradyrhizobium sp.* was the dominant genus in both zones, as well as, *Paraburkholderia sp.*, *Pseudomonas sp*. and *Massilia sp.*

Isotopic analysis revealed that nitrogen provided by fixation is being used by young plants regarding values of $\delta^{15}N$ in leaves, however biological nitrogen fixation is not functioning regarding values from young plants nodules.

Our study shows that, after fire, there seems to exist some bacteria as first settlers that are predominantly nitrogen-fixing bacteria; *Bradyrhizobium sp.* was revealed as an extremely important genus in this symbiosis.

Keywords: *Acacia longifolia*, nodules, fire, nitrogen, *Bradyrhizobium sp.*

Resumo

As invasões biológicas são uma das maiores preocupações atuais no que diz respeito à biodiversidade local, uma vez que as espécies exóticas, para garantir a sua sobrevivência no novo *habitat* natural, acabam por superar as espécies nativas. Esta dicotomia nativas-exóticas deve ser abordada de forma dinâmica e pragmática, do ponto de vista de conservação das espécies, aliado ao facto do planeta estar em constante e rápida mudança. Quando invasoras, as espécies exóticas têm diversos efeitos no novo *habitat* que podem variar com o tempo e exponencialmente com as alterações climáticas; são consideradas uma das mais sérias ameaças à diversidade e funcionamento dos ecossistemas e são descritas como "animais ou plantas que são introduzido(a)s, acidental ou deliberadamente num *habitat* natural, no qual não são normalmente encontrad(o)as, acarretando graves consequências neste seu novo ambiente".

Acacia longifolia (Andrews) Willd. é um exemplo extremamente relevante de uma espécie exótica invasora, pertencendo à terceira maior família de angiospérmicas, as leguminosas (Fabaceae ou Leguminoseae) e apresentando uma distribuição global em diversos *habitats* desde as florestas tropicais às regiões áridas. *A. longifolia* é nativa dos sistemas dunares das zonas costeiras do sul da Austrália, tendo sido introduzida em Portugal para estabilização das dunas, combate à erosão e com fins ornamentais durante o final do século XIX e início do século XX; no entanto, tornou-se uma grande questão ecológica, tendo sido classificada como uma invasora generalizada com uma elevada plasticidade adaptativa, sendo inclusivamente descrita como "engenheira de ecossistemas".

Recentemente, esta espécie é classificada como uma das invasoras mais agressivas, não só em Portugal como na África do Sul, Califórnia e Brasil, devido ao facto de (1) reduzir a biodiversidade das comunidades locais devido ao desenvolvimento de uma copa densa e capacidade de se comportar como arbusto ou árvore, (2) impacto nos serviços dos ecossistemas devido à elevada utilização de recursos hídricos, (3) alterar a dinâmica dos ciclos de nutrientes nomeadamente através da fixação simbiótica de azoto e (4) alterar os regimes de fogos devido à excessiva acumulação de folhada. Em Portugal, em sistemas dunares, tem vindo a ser observado o impacto da *A. longifolia* desde a fase inicial do processo de invasão, no qual as espécies nativas demarcam a sua influência através do acréscimo de azoto.

Nos climas mediterrânicos, como é o caso de Portugal, os fogos, que ocorrem frequentemente, promovem a germinação das sementes de *A. longifolia*, no entanto, o sucesso colonizador desta espécie é potenciado pela sua associação simbiótica com bactérias fixadoras de azoto que garantem o seu sucesso em ecossistemas com limitação de nutrientes e facilitam o desenvolvimento, sendo designadas como bactérias que promovem o desenvolvimento da planta. De facto, esta simbiose tem vindo a ser estudada como modelo, devido aos desafios que acarreta para as plantas, que necessitam de se habituar à presença das bactérias sem inviabilizar a sua

sobrevivência e para as bactérias, cujo *fitness* é largamente incrementado. Concomitantemente, esta simbiose implica uma relação íntima entre ambas as partes, a fim de se desenvolver o mutualismo na sua forma funcional e crucial: a fixação do azoto, através da qual as plantas recebem o azoto sob a forma de amónia e as bactérias recebem carboidratos e proteção. Este processo de fixação de azoto decorre em estruturas diferenciadas nas raízes da planta, denominadas como nódulos. A nodulação é um processo complexo de troca de sinais e reconhecimento celular entre a planta (macrosimbionte) e as bactérias compatíveis (microsimbiontes), para que o processo de infeção e subsequente diferenciação em bacteróides ocorra e a fixação inicie.

Os procariotas fixadores de azoto, também conhecidos como diazotróficos, são um grupo altamente diverso, tanto taxonómica como funcionalmente, incluindo bactérias de diversas classes: Alphaproteobacteria, onde se incluem os géneros *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* e *Ensifer* (anterior *Sinorhizobium*), Betaproteobacteria, que inclui o género *Burkholderia* (recentemente descrito como *Paraburkholderia* quando associado às plantas) e Actinobacteria como o género *Frankia*. *A. longifolia* estabelece simbiose, maioritariamente com os géneros de Alphaproteobacteria mencionados. Estes procariotas são comummente designados de rizóbios.

Este estudo teve como principais objetivos (1) descrever o(a)s géneros/espécies bacteriano(a)s estão envolvido(a)s nesta simbiose, (2) analisar se o fogo influencia/altera a comunidade bacteriana que é estabelecida nos nódulos e (3) descrever o ambiente em que os nódulos se inserem, bem como, a sua caracterização morfológica.

Nódulos de plantas jovens de *A. longifolia* (20-60 cm) foram recolhidos no campo em zonas não ardidas e ardidas (um ano após o fogo). Após aplicação de um protoloco de desinfeção, os nódulos foram macerados em *pools* de 1-4 nódulos e fez-se crescimentos em meio YMA; para estudos morfológicos e estruturais, foram feitas secções longitudinais e transversais para observação à lupa.

O *DNA* bacteriano foi extraído das culturas puras obtidas e procedeu-se ao *fingerprinting* por amplificação por PCR com os *primers* universais M13 e GTG-5. A análise da diversidade teve por base a construção de dendrogramas utilizando o coeficiente de similaridade de Pearson e o método de aglutinação UPGMA. A amplificação do rRNA 16S, após análise de *clusters*, permitiu identificar géneros (e espécies) envolvidos nesta simbiose. Adicionalmente, aplicou-se, dum ponto de vista percursor e exploratório, a Sequenciação de Nova Geração através do sistema *Nanopore* para complementar os dados obtidos relativamente à diversidade bacteriana pela metodologia anterior. Paralelamente, a caracterização do solo e análises isotópicas das folhas e dos nódulos relativamente ao carbono e azoto foram feitas para compreender o ambiente exterior à simbiose e o microambiente do nódulo, respetivamente.

Um total de 152 isolados foi obtido, 92 isolados a partir de nódulos da zona não ardida e 60 obtidos da zona ardida. Uma maior diversidade foi observada na zona não ardida comparativamente à zona ardida, no entanto, uma maior especificidade bacteriana, considerando a capacidade de fixar azoto, parece estar associada à zona ardida. Isto poderá indicar que o fogo tem influência no "bacterioma" do nódulo.

Bradyrhizobium sp. foi o género mais representado (e dominante) em ambas as zonas, seguido dos géneros *Paraburkholderia sp.* e *Pseudomonas sp*. Estes resultados, obtidos através de *fingerprinting* e sequenciação da região 16S foram corroborados pelos dados obtidos por Sequenciação de Nova Geração, adicionando um outro género, *Massilia sp.*, que também apresentou elevada representatividade na zona não ardida. A nível intraespecífico, verificou-se uma grande diversidade de espécies de *Bradyrhizobium sp.*, representadas em ambas as zonas, destacando-se o *Bradyrhizobium cytisi*. Isto parece sugerir que decorre um processo de especialização simbiótica entre a *A. longifolia* e esta espécie, podendo ser este o fator-chave para garantir a fixação eficiente de azoto, desempenhando um papel facilitador.

As análises isotópicas revelaram a ausência de fixação biológica de azoto, como indicaram os valores do $\delta^{15}N$ dos nódulos; contrariamente, estes valores para as folhas corroboram a utilização de azoto obtido por fixação no desenvolvimento da planta. Isto parece evidenciar que a nodulação é um processo dinâmico e não permanente.

A. longifolia é uma típica invasora, uma vez que tem a capacidade de se adaptar a diferentes condições como a seca, escassez de nutrientes e perturbações. O fogo parece ter influência na diversidade bacteriana encontrada dentro dos nódulos e na sua função de fixação de azoto. Após esta perturbação, *A. longifolia* parece preferenciar as bactérias fixadoras de azoto, podendo estas ser descritas como as "primeiras colonizadoras" aquando restabelecimento da simbiose, nas quais se incluem diferentes espécies de *Bradyrhizobium sp*. Um maior conhecimento desta relação simbiótica pode contribuir para a melhor gestão e controlo desta espécie exótica invasora.

Keywords: *Acacia longifolia*, nódulos, fogo, azoto, *Bradyrhizobium sp.*

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List of Abbreviations

AON - Autoregulation Of Nodulation
BLAST - Basic Local Alignment Search Tool
BNF - Biological Nitrogen Fixation
bp - base pairs
BZ - Burnt Zones
C - Carbon
cm - centimeter
DNA - DeoxyriboNucleic Acid
dNTP – DeoxyNucleotide TriPhosphate
EDTA - EthyleneDiamineTetracetic Acid
EPS - ExoPolySaccharide
Fig. - Figure
GES - Guanidium thiocyanate, EDTA and Sarkosyl
h - hour
HGT - Horizontal Gene Transfer
IAS - Invasive Alien Species
K - Potassium
kb - thousand of base pairs (kilobase)
KCl - Potassium chloride
KOH – Potassium hydroxide
K_2O - Potassium oxide
LGT - Lateral Gene Transfer
$MgCl_2$ – Magnesium chloride
min - minute
mm - milimeter
N - Nitrogen
N_2 - Atmospheric nitrogen
N_2O - Nitrous oxide
NF - Nod Factor
NGS - Next Generation Sequencing
NH_3 - Ammonia
NH_4^+ - Ammonium ion
NO - Nitric oxide
NO_2^- - Nitrite
NO_3^- - Nitrate
NRE - Non-Rhizobial nodule inducing bacterial Endophytes
OTU - Operational Taxonomic Unit
OVF - Optimal Value Found
P - Phosphorus
PBM - PeriBacteroid Membrane
PCR - Polymerase Chain Reaction
PGPB - Plant Growth Promoting Bacteria
PGPR - Plant Growth Promoting Rhizobacteria
P_2O_5 - Phosphorus pentoxide
rpm - rotations per minute
rRNA – ribosomal RiboNucleic Acid

SIIAF - Stable Isotopes and Instrumental Analysis Facility
SOM - Soil Organic Matter
TBE - Tris Borate EDTA
TES - Tris-EDTA and SDS
UBZ - Unburnt Zones
UPGMA - Unweighted Pair-Group Method with Arithmetic Mean
YMA - Yeast Mannitol Agar

1. Introduction

1.1 Biological invasions: *Acacia longifolia* case

Biological invasions are a major threat in what concerns biodiversity, once non-natives, leading to its extinction and modifying natural environments, outgrow native species. These non-native species can also be defined as exotic or alien and this dichotomy native-alien is an issue, which entails more dynamic and pragmatic approaches to the conservation of species, considering a fast-changing planet. Alien species can have several effects in the new habitat, which vary with time and exponentially with environmental changes (Davis *et al.*, 2011). Alien species can pass through a naturalization process, which leads to its invasion, and this is highly related with species intensive reproduction over natives (Richardson and Pyšek, 2013).

Invasive alien species (IAS) are considered one of the most serious threats to local biodiversity and ecosystem functioning and have been defined as "animals and plants that are introduced accidentally or deliberately into a natural environment where they are not normally found, with serious negative consequences for their new environment" (EU Regulation 1143/2014).

Acacia longifolia (Andrews) Willd. (Sidney golden wattle) is an example of an IAS. This species belongs to the third largest family of Angiosperms, the Fabaceae or Leguminoseae, distributed worldwide in diverse habitats from rain forests to arid zones (Marchante, 2008). Three subfamilies are considered, namely Caesalpinioideae, Papilionoideae and Mimosoideae, where *Acacia spp*. are included.

A. longifolia is an exotic species, native from coastal dunes of southern Australia, introduced in Portugal for dune stabilization, preserve sand erosion and ornamental purposes during the late 19th century and the beginning of the 20th (Peperkorn *et al.*, 2005); however, nowadays it is an ecological issue, because it became a widespread invader with a high adaptive plasticity, being inclusively described as an "ecosystem-engineer" (Stock *et al.*, 1995; Marchante *et al.*, 2004, 2008, 2009; Yelenik *et al.*, 2007). This "engineering" of the new ecosystem stems from high growth rates, production of highly resistant, dispersible and fire stimulated seeds, absence of natural enemies, ability to fix nitrogen and water consumption (Marchante *et al.*, 2003; Antunes *et al*., 2018). In Portugal, in sand dunes, it has been observed a large ecological impact of *A. longifolia* at an early stage of the invasion process, where neighboring native species were influenced by additional nitrogen supply (Rascher *et al.*, 2012; Hellman *et al*., 2011; Ulm *et al.*, 2017). Moreover, this species is classified as one of the most aggressive invaders, not only in Portugal but also in South Africa, California and Brazil. *A. longifolia* (1) reduces the local communities' biodiversity due to its very dense canopies and ability to behave as a tree or shrub, (2) impacts ecosystem services due to high water and resources' utilization, (3) alters nutrient

cycles dynamics mainly through symbiotic nitrogen fixation (Ulm *et al.*, 2017; Meira-Neto *et al.*, 2018) and (4) impacts the fire regimes through the huge accumulation of litter in soils (Marchante *et al*., 2008; Werner *et al*., 2008). All together, these characteristics, highlighting symbiotic interactions, allow *A. longifolia* to succeed in nutrient-limited ecosystem, being over-represented among invaders (Marchante *et al.*, 2003).

Symbiosis had been studied as a model when it occurs between plants and microorganisms, due to the challenges for (a) plants to overcome the presence of bacteria without fail on surviving and (b) bacteria, which had significant improvements in its fitness within the rhizospheric environment (Sachs *et al*., 2018). For this to occur, an intimate relation allowing a gain for both organisms involved is implied, reason why it is called mutualism (Moran *et al.*, 2006). This relation is very common between legumes and bacteria, in a very distinct and priceless way: nitrogen fixation.

1.2 Nitrogen fixation: a part of nitrogen cycle

Nitrogen (N) is an abundant element in the atmosphere, however, despite its 78 % abundance as N_2 (stable form), it is essentially non-utilizable by most organisms, mostly because of its covalent triple bond between N atoms which makes it difficult to break. However, it is, for all known living beings, an essential element, taking part in such a variety of processes and in the basic building structures of some molecules such as nucleic acids and proteins, including enzymes (Howard and Rees, 1996).

Fortunately, there are numerous nitrogen-fixing prokaryotes able to break this bond and reduce N_2 to available fixed forms like ammonia (NH_3), a process called biological nitrogen fixation (BNF), making the nitrogen cycle one of the most important biogeochemical cycles worldwide that contributes to almost half of the nitrogen biologically available (Lee and Hirsch, 2006; Lira MA Jr. *et al*., 2015). Different microorganisms take a role in this biologically controlled cycle that responds actively to changes in redox states and oxygenation of the atmosphere. N_2 is fixed only by microorganisms so far and through nitrogenase.

Nitrogenase is an oxygen-labile metalloenzyme responsible for redox reactions in nitrogen fixation step in nitrogen cycle (Anbar and Knoll, 2002); it is composed by dinitrogenase and dinitrogenase reductase. Both subunits are composed by iron and the first one is also composed by molybdenum. In fact, it is due to this iron-molybdenum center, containing the iron and molybdenum cofactor, that N_2 reduction is possible. Furthermore, once N_2 has that triple bond between N atoms, it is an extremely dispending energetic process, so six electrons must be transferred to reduce it. The subsequent reductions occur without accumulated intermediates directly through nitrogenase. The fixed forms of N_2 can be NH_4^+ (ammonium ion), the major form

in anoxic systems and NO_3^- (nitrate), the dominant in oxic systems (Madigan and Martinko, 2006) (Fig. 1).

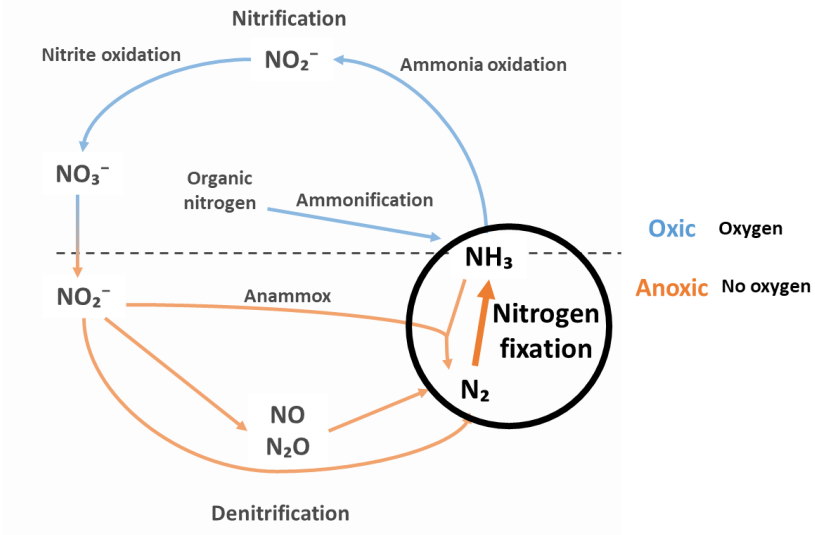


Figure 1 – Nitrogen cycle adapted from Madigan and Martinko (2006).

1.2.1 Nodules: the "stage" of the symbiosis

Biological nitrogen fixation is one the most relevant abilities of *A. longifolia*, through its symbionts. This relationship between legumes and rhizobia evolved approximately 58 million years ago, occurring in 88 % of all legume species (Sachs *et al.*, 2011; Sprent, 2007). This process is achieved inside structures formed *de novo*, called nodules, developed on roots through the infection of bacteria (Dupont *et al.*, 2012). Nodules formation include a complex reciprocal signal exchange and cellular recognition between the plant and the compatible bacteria. In fact, besides some exceptions, it is known that the involved bacteria can develop nodules only with a restrict number of hosts, and the reciprocal is also true: each host accept a restricted number of bacteria as microsymbiont partners (Caetano-Anollés and Gresshoff, 1991).

Flavonoid compounds released by macrosymbiont attract the microsymbiont and stimulate the production of Nod factors (NFs) that functions as a trigger to the signal cascade that leads to the organogenesis of nodules (Ferguson *et al.*, 2010). This specific signalization step is crucial for the initiation of nodule development and bacterial entry (Madsen *et al.*, 2003; Radutoiu *et al.*, 2007). Furthermore, a successive differentiation process occurs, and each bacterium is involved with plant cell membrane, the peribacteroid membrane (PBM) and both form symbiosomes; inside it, the differentiation of bacteroids - nitrogen-fixing active form - occurs. Through repeated endoreplication cycles, infected cells increase and symbiosomes became large polyploid cells hosting thousands of bacteroids. Mature nodules remain active until senescence due to aging or stress (Jones *et al.*, 2007; Kondorosi *et al.*, 2000).

Nodule organogenesis and plant gene expression are interdependent, which is implicit for a symbiosis relationship (Benedito *et al.*, 2008; El Yahyaoui *et al.*, 2004) (Fig. 2).

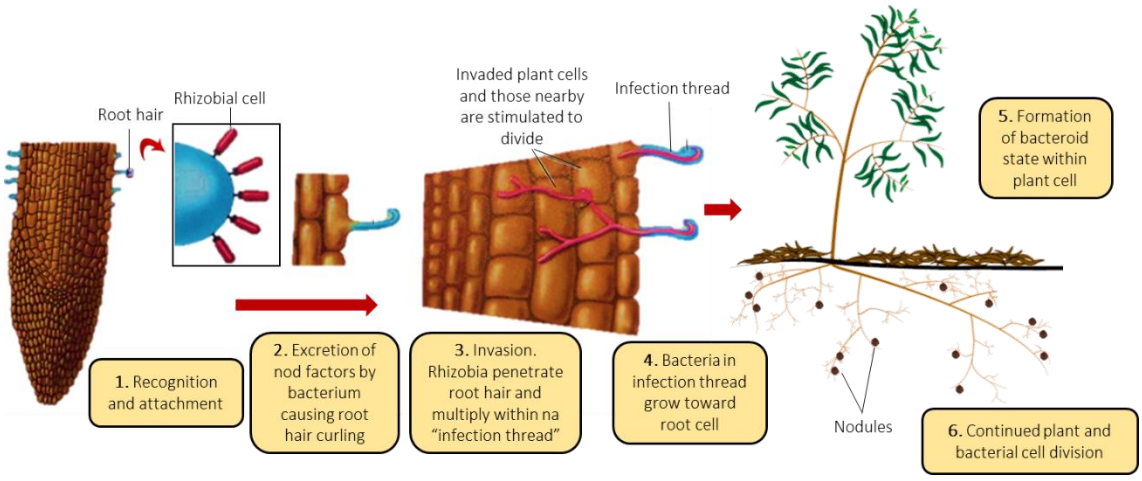


Figure 2 – Steps in the formation of the root nodule in legume infected by rhizobia. Adapted from Madigan and Martinko (2006).

Besides structural development ending in nodule formation, it is also necessary the presence of nitrogenase with optimal activity under microaerophilic conditions, that are created inside nodules by an equally important protein, leghemoglobin. Interestingly, the expression of nodule-specific plant genes accompanies nodule development allowing leghemoglobin's accumulation in the cytoplasm that works as an oxygen-buffer, essential to maintain the oxygen flux to respiration (Ott *et al.*, 2005).

When everything is perfectly settled, symbiosis is working: plant is gaining nitrogen fixed forms, whilst bacteria is gaining carbohydrates and protection (Ferguson *et al*., 2019).

1.2.1.1 Rhizobia: the microsymbionts

Nitrogen-fixing prokaryotes, also known as diazotrophs, play a key role in BNF and are abundant in soils, taking part of the rhizosphere communities, which are highly diverse, both taxonomically and functionally (Kamutando *et al.*, 2018). This group of prokaryotes include free-living bacteria both aerobes and anaerobes and symbiotic bacteria that require a host for BNF process. In this last group, there are included bacteria mainly from Alphaproteobacteria, such as *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Ensifer* (previously *Sinorhizobium*) genera, Betaproteobacteria, which includes *Burkholderia* genera (recently described as *Paraburkholderia* once associated with plants), and Actinobacteria like *Frankia* (Madigan and Martinko, 2006;

Long, 1996). These bacteria can also be named as “plant growth promoting bacteria” (PGPB) or even “plant growth promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova, 2009).

Regarding *Acacia spp.* microsymbionts already described, they fit amongst the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Lafay and Burdon, 2001; Marsudi *et al.*, 1999; Nick *et al.*, 1999a and b), that have a great diversity in what concerns host range, symbiotic effectiveness and geographic origins (Leary *et al.*, 2006).

1.2.1.2 *A. longifolia*: the macrosymbiont

A. longifolia, as the host plant, is responsible for attracting the bacteria present in soils and can control the number of nodules formed, once nodulation is a costly process. This is the reason why Ferguson *et al.* (2019) concludes that the host controls the party. In fact, an inherent pathway of autoregulation of nodulation (AON) can be depicted, that responds to infection in order to maintain the symbiotic system working optimally (Caetano-Anollés and Gresshoff, 1991; Delves *et al.*, 1986; Ferguson *et al.*, 2010; Kosslak and Bohlool, 1984; Reid *et al.*, 2011). Furthermore, nitrogen fixation is part of a regulation pathway that works in feedback, according to the nitrogen available in the environment, ultimately saving resources (Lim *et al.*, 2014; Reid *et al.*, 2011).

Concurrently, in such a highly taxonomic and functional diversity present in rhizosphere communities (Rodríguez-Echeverría *et al.*, 2007), *A. longifolia* takes advantage due to its promiscuity, which increases the probability to find partners and ensure symbiosis success. For this reason, when a plant species is under an adaptation process, with a symbiotic association involved to overcome nutrient necessities and the assurance of physiological development, promiscuity can be an important factor (Rodríguez-Echeverría *et al.*, 2011).

1.3 Fire as a challenging factor

Fires, both natural and anthropogenic, are one of the most common perturbations present in natural ecosystems that can alter the landscape and interact with plant–soil biota feedbacks, thereby influencing plant invasions (Blumenthal, 2005; Kulmatiski and Kardol, 2008). On one hand, natural fires have a great impact in Mediterranean landscape, on the other hand, this perturbation is one of most frequent management tools that have been subjected to human use (Pausas and Vallejo, 1999). Mediterranean climate is distinguished by dry and hot Summers and wet and mild Winters (Gams *et al.*, 1993) and occurs in Portugal and in other regions like California, Chile, Australia and South Africa. In fact, it is considered one of the factors responsible for the unique characteristics of these regions (Blondel and Aronson, 1999).

Despite vegetation damage, fires cause alterations on soils through nutrient losses by volatilization, especially carbon, nitrogen and sulfur, for a few weeks after (Raison *et al.*, 1985) and some modifications due to the scarce vegetation cover (wind and water erosion and nutrient

lixiviation) immediately after fire extinction and until vegetation recovers (DeBano *et al*., 1976; Khanna and Raison, 1986). This disturbance influences such a variety of physical and chemical properties of the soil, including modifications in structure itself, loss or reduction of soil organic matter (SOM) and pH increase (Certini, 2005). It can also have indirect consequences like hydrophobicity increase through the formation of a water repellent layer which leads to the decrease of infiltration and consequently to the increase of runoff and erosion (DeBano, 2000).

In the Mediterranean region, erosion after a fire is very frequent ascribed to Autumn rains (Pausas and Vallejo, 1999). After fire, there is a natural reconversion in plant communities and the proliferation of exotic species can be triggered out (Marchante *et al.*, 2003). In addition, once there is no competition with other species, the availability of limiting resources increase following perturbation, which may favor the success of fast-growing exotic species like *A. longifolia*, which forms dense stands due to pyrostimulated seeds that existed in the seed bank, seriously threating the native plant communities (Carvalho *et al.*, 2010).

2. Objectives

Considering that *A. longifolia* is one of the most prominent and widespread invaders in Portugal with a huge impact in soils ascribed to BNF and adding the fact that fire is preponderant in its seed germination, growth and adaptation, it is important to understand what could be the role of fire in symbiosis establishment.

With that in mind, the main objectives were to understand (1) which bacteria (genus/species) are present in the symbiosis; (2) if fire will alter the bacterial community established in the nodules; and (3) nodule morphological characterization.

Ultimately, this will help to understand the traits of an alien species responsible for its competitiveness, providing relevant information that will contribute to its knowledge of the dichotomy between the invasive plant and its interaction with the environment under changing.

3. Material and Methods

3.1 Plant collection sites

Young *A. longifolia* plants (about one-year old) were collected in Mira, Aveiro, Portugal mainland. This region has a Mediterranean climate, with an Atlantic influence, with a mean annual precipitation of 920 mm, and mean monthly temperatures ranging from 10.2 ºC in January to 20.2 ºC in June (Rodríguez-Echeverría, 2010). Considering that fire occurred on 17th of October 2017, six sites were selected and sampled in October 2018 (one year after fire): three unburnt zones or zones where no fire occurred (UBZ) (40.52451º, -8.67253º; 40.52451º, -8.67253º; 40.53130º, -8.67603º) and three burnt zones (BZ) (40.526482º, -8.673788º, 40.52546º, -8.67437º; 40.53014º, -8.675279º) (Fig. 3 and Appendix 1).

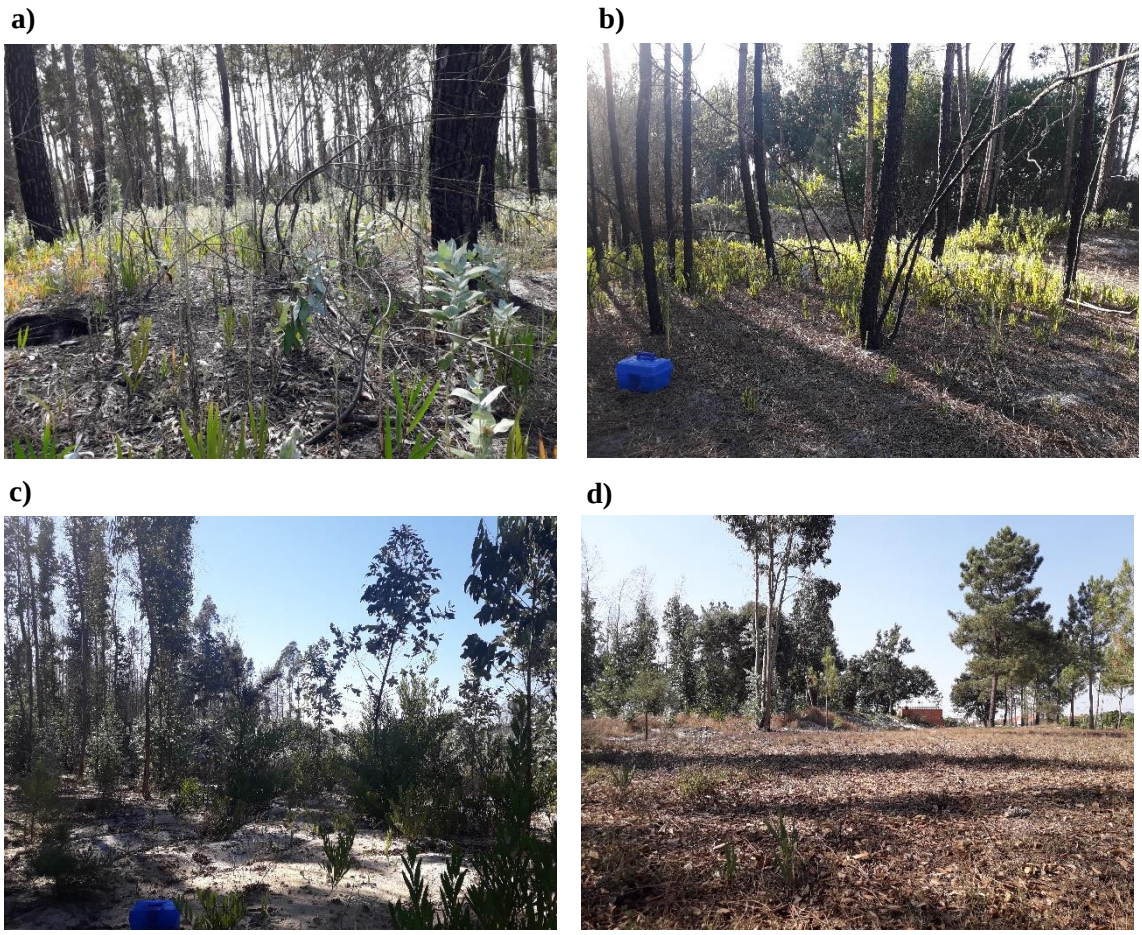


Figure 3 – Example of the sampled sites: a) and b) are burnt zones (one year after fire); c) and d) are unburnt zones.

3.2 Soil characterization

Soil samples were collected from the six sampled zones (Fig. 4) from a depth of 0–20 cm upon removal of the litter layer, and each sample consisted of approximately 1.5 kg. A mixed sampled were made through the collection of soil from three spots in each zone (Sankhla *et al*., 2017). Soil was analyzed for basic characteristics such as texture and particle size, pH (water and KCl 1 M), organic matter, P and K and the amount of total and mineral N ($N\text{-}NH_4^+$ and $N\text{-}NO_3^-$). Analysis were conducted by the Plants and Soils Laboratory from Universidade de Trás-os-Montes e Alto Douro (UTAD), Portugal.

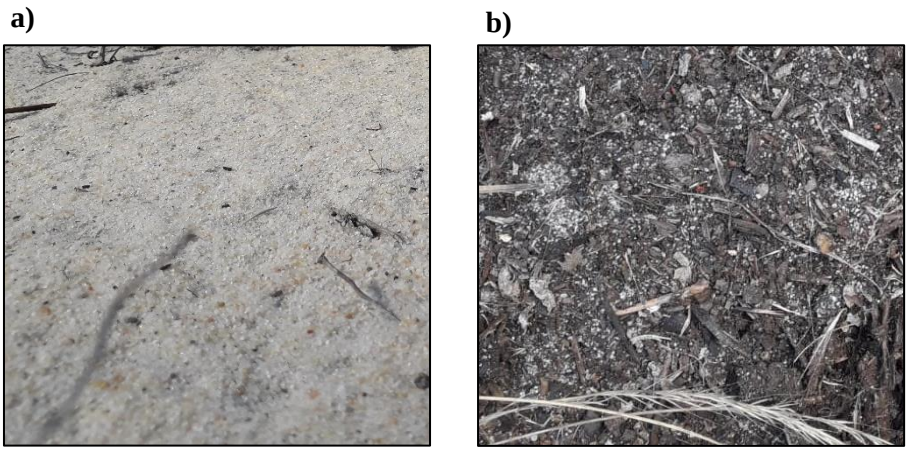


Figure 4 – Soils observed in the field from a) unburnt zone and b) burnt zone.

3.3 Nodules collection

All the nodules from eight individual *A. longifolia* young plants (20-60 cm) were collected in each six sampled zones (three unburnt and three burnt zones), by digging up plants to locate root segments with attached nodules (Fig. 5a, c and d). Nodules were stored in Falcon tubes with silica gel and kept under room temperature until use (Fig. 5b).

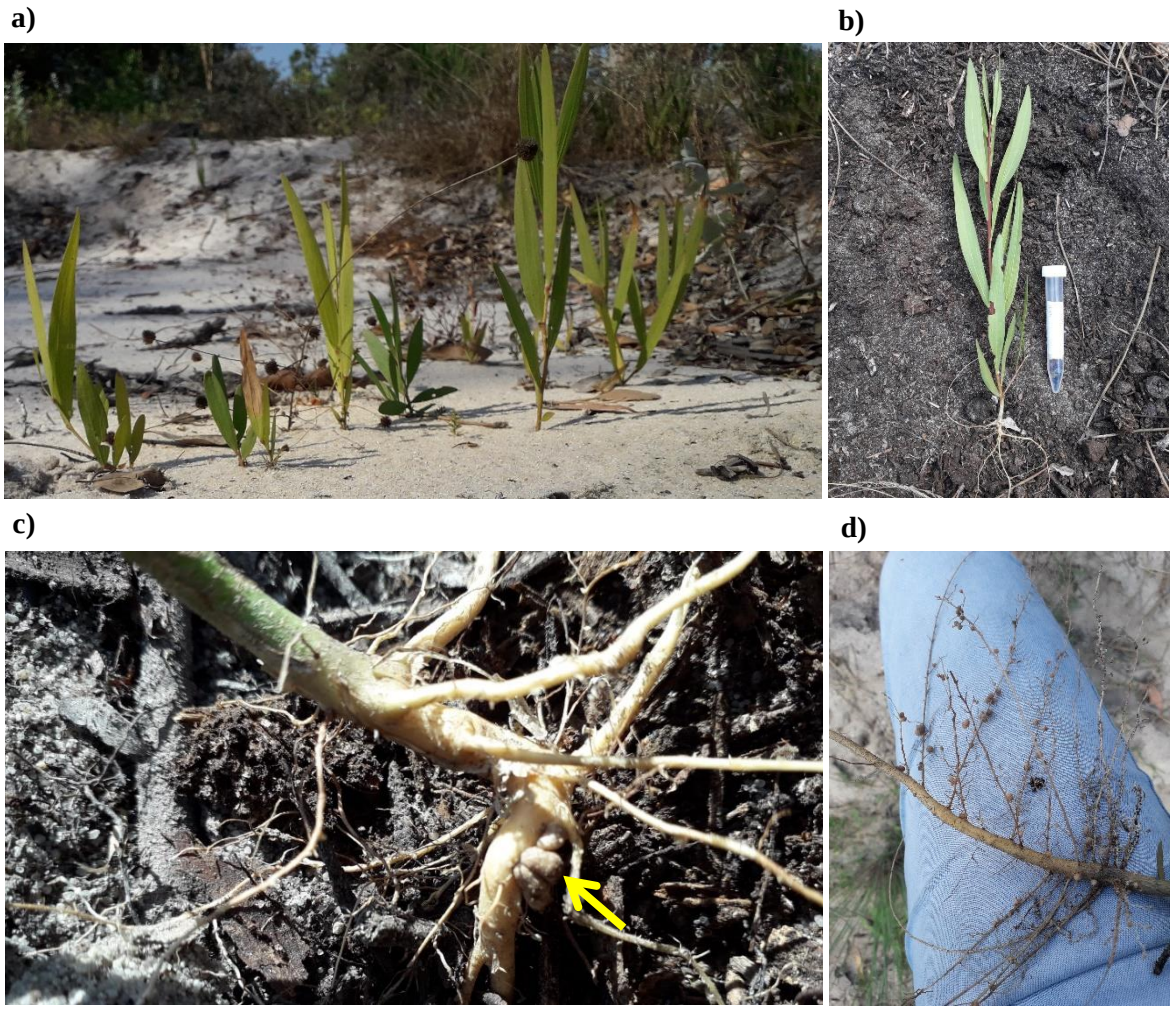


Figure 5 – *A. longifolia* young plants with different sizes growing in the field (a), one-year old plant (b), nodules found in the roots (c) and root with a huge number of nodules (d). The arrow highlights nodules growing in roots.

3.3.1 Nodules measurements and morphology

A. longifolia nodules were measured and organized by size.

Cross and longitudinal sections were performed to study morphology, internal structure and organization. For the morphological study, nodules were hydrated for 12 hours (h) and sections were obtained with a razor blade. For the internal structure, nodules were fixed in 2.5 % glutaraldehyde in 0.05 M cacodylate buffer and washed three times in water. Longitudinal and cross sections (10 µm thickness) were obtained with a freezing microtome (BRIGHT 5040 Rotary Retracting Microtome with solid state freezer stage), mounted on glass slides and stained with different dyes (Lugol, toluidine blue, Sudan red, and lactophenol and blue cotton). Images were obtained with Zeiss Lumar V12 Stereoscope.

3.4 Bacterial isolates

3.4.1 Growth media and isolation

Yeast Mannitol Agar (YMA) (Appendix 2) was used for isolation and maintenance of bacterial isolates. For initial isolation from nodules, cycloheximide (0.01 %) was added in media to inhibit fungal growth (Vincent, 1970). Subculturing steps were performed in YMA without cycloheximide. All steps of bacteria isolation and cultivation were performed on a laminar flow chamber.

For bacterial isolation, nodules were rehydrated in water during 12 h and surface-disinfected in 70 % ethanol for 1 minute (min), then transferred to commercial sodium hypochlorite for 6 min, 1 min in 70 % ethanol, followed by six washes in sterile distilled water (Fig. 6). For disinfection control, nodules were dried with sterile filter paper and then rolled (surface printing) in YMA plates and incubated at 28 ºC for four days. In the absence of growth, pools of 1-4 nodules were crushed in 500 µL of 0.85 % sodium chloride; by serial dilutions method, the obtained suspension was inoculated on YMA supplemented with cycloheximide (0.01 %) and incubated at 28 ºC for 12 days. A total of 95 nodules were studied and a total of 152 isolates were obtained. Pure cultures were obtained with three or more subculturing steps using standard protocols (Cappuccino and Sherman, 1998; Crisóstomo *et al*., 2013).

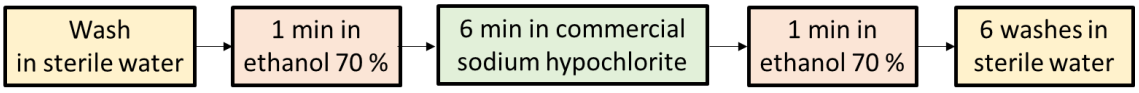


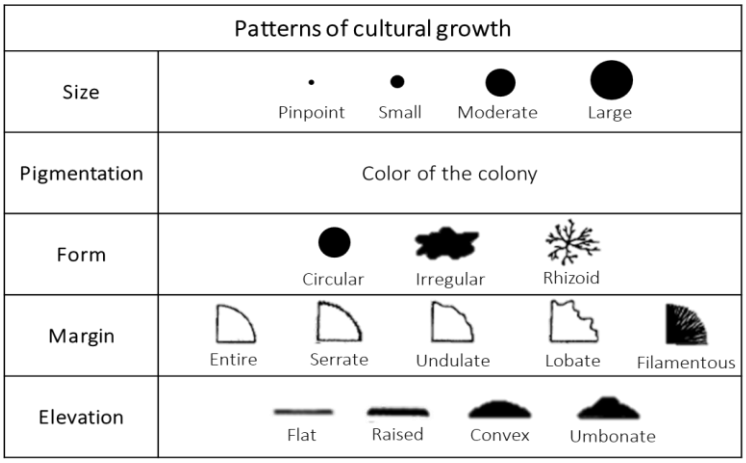
Figure 6 - Protocol used for nodules disinfection. Adapted from Vincent (1970).

3.5 Phenotypic studies

3.5.1 Colonies' characteristics

Macroscopic appearance of bacteria' growth was analyzed. The patterns of growth considered were described for nutrient agar plates, including the following parameters: Days of growth until the appearance of visible colonies, Size (Pinpoint, Small, Moderate or Large), Pigmentation (Color of the colony), Form (Circular, Irregular or Rhizoid), Margin (Entire, Lobate, Undulate, Serrate or Filamentous) and Elevation (Flat, Raised, Convex or Umbonate) (Table 1).

Table 1 – Macroscopic appearance of microorganisms' colony growth adapted from Cappuccino and Sherman (1998).



3.5.2 Bacteria morphology and characterization tests

Bacteria were classified as coccus or rods. These two morphologies may appear isolated, in strings, in aggregates or in pairs, which receive specific designations.

Routine tests were performed to cluster the colonies according to the results: Gram staining and KOH test, catalase test and oxidase test. Each of these tests has a dichotomic response, positive (+) or negative (-). All these tests were performed after 24 h of visible colonies growth.

3.6 Molecular studies

3.6.1 DNA extraction

For DNA extraction using GES (Guanidium thiocyanate, EDTA and Sarkosyl) modified protocol, one loop of colonies (10 µL) from each isolate were suspended in 250 µL of lysis buffer (Tris-EDTA and SDS) with approximately 100 µL of microspheres. For mucous colonies, five washes in sterile water were done through suspension in 1 mL of sterile water before adding the lysis buffer and microspheres. The protocol was followed, and the extracted DNA was maintained in 100 µL of 1x TE buffer (Tris-EDTA) (Appendix 3).

3.6.2 PCR-Fingerprinting

3.6.2.1 Polymerase Chain Reaction (PCR) and running

Bacterial DNA from all isolates was amplified through Polymerase Chain Reaction (PCR) using the universal primer M13 and some of them were amplified with GTG-5. It was performed using a final volume of 25 µL containing the final concentrations: 50 ng of template DNA, 1 U of Taq DNA polymerase (Invitrogen), 25 pmol of the primer, 3 mM of $MgCl_2$, 0,2 mM of each dNTPs and 1x PCR buffer. The PCR temperature profiles were 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 50 °C for 2 min, 72 °C for 2 min and a final extension at 72 °C for 5 min.

The amplified PCR products were separated by electrophoresis on 1 % (w/v) molecular biology agarose gel dissolved in 0.5x TBE buffer. In each well, 5 µL of each sample were applied, and 5 µL of the 1 kb plus DNA ladder, to estimate the molecular weight of DNA fragments. This ladder is composed by 20 DNA bands spanning from 100 to 12000 base pairs (bp) (Sankhla *et al*., 2017).

Gels ran at 85 V for 5 h. After running, they were stained in 0.5 µg. mL^{-1} ethidium bromide for 10 min, washed in water to remove excess staining and visualized under UV light in a transilluminator with the software Alliance 4.7 (Uvitec, Cambridge).

3.6.2.2 Cluster analysis with BioNumerics

After DNA migration, band profiles obtained in the agarose gel were compared using BioNumerics. A cluster analysis as a dendrogram was performed using the unweighted pair-group method with arithmetic mean algorithm (UPGMA) and the Pearson correlation coefficient.

Reproducibility was analyzed based on a 10 % replicate of the total isolates, in order to establish the cut-off level. This allows to conclude that a similarity lower than that level between two isolates supports its difference. The cut-off level applied in this study was 84 %, so all the isolates below this similarity threshold were considered different.

After applying the cut-off, dendrogram was optimized, considering that the bands chosen to represent the profile of an isolate come from different gels, it is of outmost importance to optimize the shift between each profile, once the analysis is based on that comparison. The optimal value found (OVF) were 1.5 % that was applied to the dendrogram.

To understand the resolution of the universal primer M13, redundancy in isolations were analyzed and PCR fingerprinting based on GTG-5 primer amplification was included for the isolates obtained from the same pool of nodules in each zone, that has a similarity above the cut-off level. Through this redundancy level, it is possible to determine the internal reproducibility and with the application of GTG-5, it is possible to understand the limitation of M13 primer in resolving two isolates.

Shannon-Wiener and Simpson diversity indexes and Pielou evenness index were used to calculate the diversity and evenness of the bacterial isolates of unburnt and burnt zone (Appendix 4).

3.6.3 16S gene amplification, purification and sequencing

After discriminating isolates by DNA fingerprinting, the 16S rRNA gene was amplified using two different primers combinations: PA(8f) with 907r and 104f with 1392r (Appendix 5) based on the *Escherichia coli* numbering system (Marchesi *et al.*, 1998). A total of 45 isolates were selected for this PCR sequencing, representing almost all the clusters obtained in the dendrogram.

The final volume of the PCR was 20 µL containing the final concentrations: 50 ng of template DNA, 1 U of Taq DNA polymerase (SurfTaq), 25 pmol of the primer, 3 mM of $MgCl_2$, 0.2 mM

of each dNTPs and 1 x PCR buffer. The PCR temperature profiles were 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min, 55 °C for 2 min, 72 °C for 1.5 min and a final extension at 72 °C for 5 min. Amplification was confirmed through electrophoresis on 1 % (w/v) molecular biology agarose gel dissolved in 0.5x TBE buffer. Once confirmed the presence of a unique band with the correct size, PCR products were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Termo Fisher Scientific), according to the manufactures protocol; after purification, the samples were sent to STABVIDA (Caparica, Portugal) for Sanger sequencing only on reverse direction.

3.6.4 Alignments

DNA sequences obtained from the Sanger sequencing were analyzed with the software *Geneious* (Drummond *et al*., 2010), performing alignments among each other and later with data available from GenBank through BLAST. All the sequences were previously "clean", which means that the pair based that were wrong sequenced (that normally occurs in the beginning and the end of the sequence obtained) were deleted, in order to increase the quality of alignments. Each sequence was treated individually, and alignments were performed with sequences available in the GenBank. The alignments with high similarity were considered. Thought this identification, it was possible to identify other isolates by comparation of similarities through dendrogram analysis.

3.7 Next Generation Sequencing (NGS)

The microbial profiling using 16S rRNA gene was performed by BioISIGenomics. A total of 10 root nodules were collected from different *A. longifolia* young plants from the six sampled sites, including the three unburnt (UBZ 1, UBZ 2, UBZ 3) and the three burnt (BZ 1, BZ 2 and BZ 3) zones. The nodules were hydrated for 12 h and then weighted. These six pools (10 nodules each) were washed with 10 mL of Washing Solution (PBS + 2 $mL.L^{-1}$ Tween 20) and placed in the orbital shaker at 140 rpm for 3 h; then, nodules were ultrasound-treated using impulses of 30 seconds, 3 times. DNA was extracted from each pool using CTAB-based DNA protocol according to Lopez (2019) and quantified using Qubit Fluorometer (v 1.01) with the Quant-IT dsDNA High Sensitivity Assay Kit. An absorbance spectrum, using a Nanodrop 1000 spectrophotometer was also obtained. PCR amplification of 16S rRNA gene and sequencing library were prepared using Oxford Nanopore Tecnologies' SQK-RAB204 kit. Amplification and barcoding were performed using ONT's 16S barcoded primers and New England Biolabs' LongAmp Taq 2x Master Mix. After amplification, samples were quantified with the same previous conditions described and then pooled to produce a sequencing library. Using FLO-MIN106 rev D flow cells on GridION X5 sequencing platform, sequencing runs were performed, and this data was acquired using the MinKNOW 19.06.8 software. Each file represented a batch of 4000 reads. Finally, generated data

was submitted on the EPI2ME 16S workflow for taxonomic classification of 16S amplicon data. All this information was provided by BioISIGenomics.

3.8 Isotopic analysis

Leaves and nodules from *A. longifolia* young plants were collected from the six sampled sites and were dried during 48 h in a drying kiln at 60 ºC. Each sample was ground using a ball mill and 2-2.5 mg were weighted for isotopic analysis. Stable isotope ratio analysis was performed at the Stable Isotopes and Instrumental Analysis Facility (SIIAF), at Faculdade de Ciências, Universidade de Lisboa, Portugal.

$\delta^{13}C$ and $\delta^{15}N$ were determined in the plant material samples using continuous flow isotope mass spectrometry (Preston and Owens, 1983), on a Sercon Hydra 20-22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas-combustion. Delta Calculation was performed according to δ= [(Rsample – Rstandard)/Rstandard] * 1000, where R is the ratio between the heavier and lighter isotopes. $\delta^{15}N$ air values are referred to air and $\delta^{13}C$ VPDB values are referred to PDB (Pee Dee Belemnite). The (secondary) reference materials used were Sorghum Flour Standard OAS/Isotope and Wheat Flour Standard OAS/Isotope (Elemental Microanalysis, UK) for nitrogen and carbon isotope ratio (with, respectively, $\delta^{15}N$ air(Sorghum Flour OAS)= 1.58+/-0.15 ‰ , $\delta^{15}N$ air(Wheat Flour OAS)= 2.85+/-0.17 ‰, $\delta^{13}C$ VPDB(Sorghum Flour OAS)= -13.68+/-0.19 ‰, $\delta^{13}C$ VPDB(Wheat Flour OAS)= -27.21+/-0.13 ‰), regularly checked against certified reference materials. Uncertainty of the isotope ratio analysis, calculated using values from six to nine replicates of secondary isotopic reference material interspersed among samples in every batch analysis, was ≤ 0.1 ‰. The major mass signals of N and C were used to calculate total N and C abundances, using Sorghum and Wheat Flour Standard OAS (Elemental Microanalysis, UK, with 1.47 % N, 46.26 % C and 1.47 % N, 39.53 % C respectively) as elemental composition reference materials. All this information was provided by SIIAF.

4. Results

4.1 Soil analysis

In our study, the soils in which *A. longifolia* young plants were collected were sandy soils and it is observed that both soils (unburnt (UBZ) and burnt (BZ)) are very similar in terms of coarse sand, thin sand, slime and clay as shown below in Table 2.

Table 2 – Mean values of granulometric analysis of soils from unburnt zone (UBZ) and burnt zone (BZ). Class of texture is also represented.

	Granulometric categories ($g.kg^{-1}$)				**Class of texture**
	Coarse sand	Thin Sand	Slime	Clay	
UBZ	912.7	50.8	9.7	26.8	Sandy
BZ	900.8	62.3	9.9	27.0	Sandy

Regarding soil analysis, it is observed that soil organic matter (SOM), P_2O_5, K_2O, total N, NO_3^- and NH_4^+ were higher in burnt zone than in unburnt zone. There were present statistically significant differences between total N values found in unburnt zone, that were lower, than the ones found in burnt one (Table 3).

Table 3 – Mean values of soil analysis: texture, water pH, Soil Organic Matter (SOM) (%), amount of P_2O_5 ($mg.kg^{-1}$), total amount of nitrogen (N) ($g.kg^{-1}$) and amount of mineral nitrogen ($mg.N.kg^{-1}$), including NO_3^- and NH_4^+. Statistically significant differences are represented by *, according to T-test with an $\alpha = 0.05$.

				Egner-Riehm Extraction		Mineral N - N $mg.kg^{-1}$	
	Texture	Water pH	SOM (%)	P_2O_5 $mg.kg^{-1}$	Total N $g.kg^{-1}$	$N\text{-}NO_3^-$	$N\text{-}NH_4^+$
UBZ	Rude	5.5	1.02	3	0.054*	2.8	2.8
BZ	Rude	5.4	2.08	16	0.124	4.3	6.3

4.2 Nodules characterization: morphology and internal structure observation

In the six sampled zones, 587 nodules were collected in three unburnt zones (UBZ) and three burnt zones (BZ) (Table 4). Although the mean number of nodules was higher in burnt zones, the differences found between each zone were not statistically significant (Fig. 7). These nodules showed different sizes and different morphologies (Fig. 8).

Table 4 – Total number of nodules collected in each zone from eight different *A. longifolia* young plants in each sampled zone (1, 2 and 3).

	Unburnt zones	Burnt Zones
1	83	168
2	128	103
3	31	74
Total	242	345

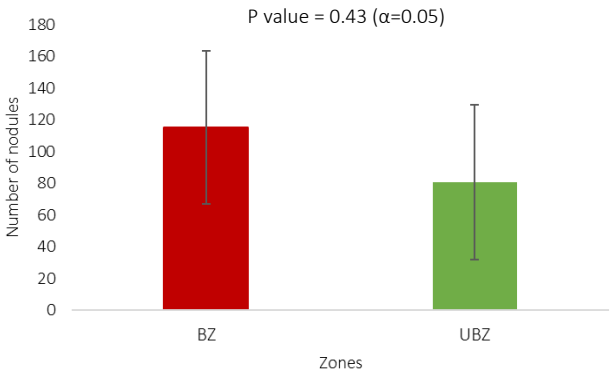


Figure 7 – Mean value (±SE) of the total number of *A. longifolia* young plants nodules from unburnt (UBZ) and burnt zones (BZ). Results for one-way ANOVA are included in the figure.

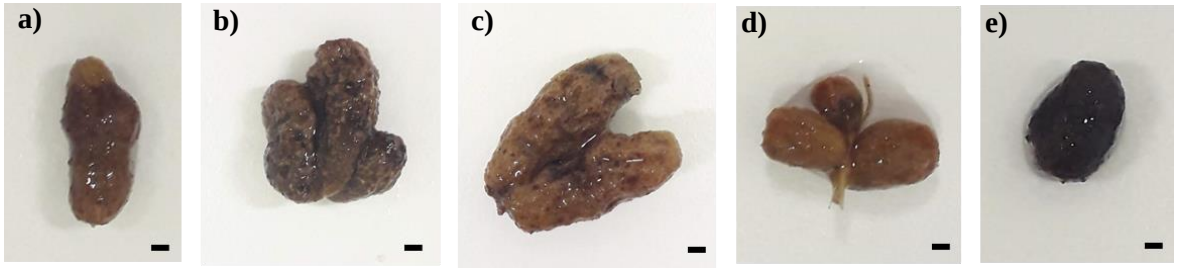


Figure 8 – *A. longifolia* young plants nodules with different morphologies a) cylindrical; b) trilobate; c) bilobate; d), e) round, both from unburnt and burnt zones. Scale bar = 1 mm.

Collected nodules from both zones presented sizes comprised between 0.1 and 1.7 cm for unburnt zones and 0.1 and 1 cm for burnt zones (Fig. 9).

Figure 9 – Example of *A. longifolia* young plants nodules collected in the field from a) unburnt and b) burnt zones and the respective size.

Longitudinal sections of *A. longifolia* nodules also revealed an inside organization in 4 different zones: meristematic zone (I), infection zone (II), nitrogen fixing zone (III) and senescence zone (IV) (Fig. 10). Nodules observation suggested the existence of nitrogen fixing zone (reddish tissues represented by III), that is shown below (Fig. 10a). Nodules with only two regions, namely meristematic zone (I) and infection zone (II) or meristematic zone (I) and senescence zone (IV) (Fig. 10b) and nodules that seemed to be destroyed or empty, with no differentiated tissues (Fig. 10c) were also observed. These different internal structures were observed in nodules collected from both zones.

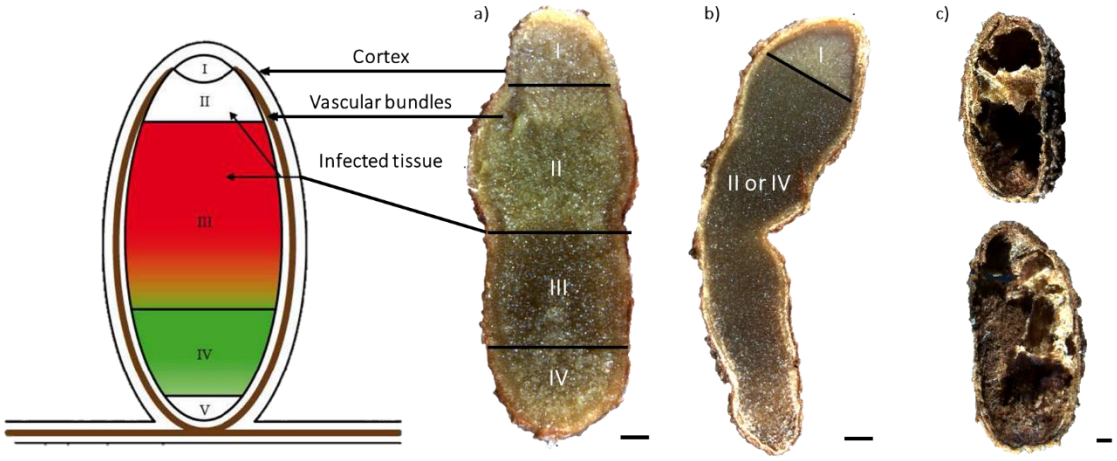


Figure 10 – Schematic representation of different mature stages of *A. longifolia* young plants nodules adapted from Franssen *et al*. (1992). a) Nodule with nitrogen-fixing zone present; b) Nodule in preliminary stage of development or before senescence; c) Destroyed nodule with any zones present. Each number represents a zone: meristematic zone (I), infection zone (II), nitrogen fixing zone (III), senescence zone (IV) and saprophytic zone (V). The images on the right were obtained with Zeiss Lumar V12 Stereoscope. Scale bar = 1 mm for a) and b); 0.5 mm for c).

Preliminary approach for studying internal morphology of *A. longifolia* young plants nodules showed that there are no differences in the anatomy of nodule (Fig. 11 and 12).

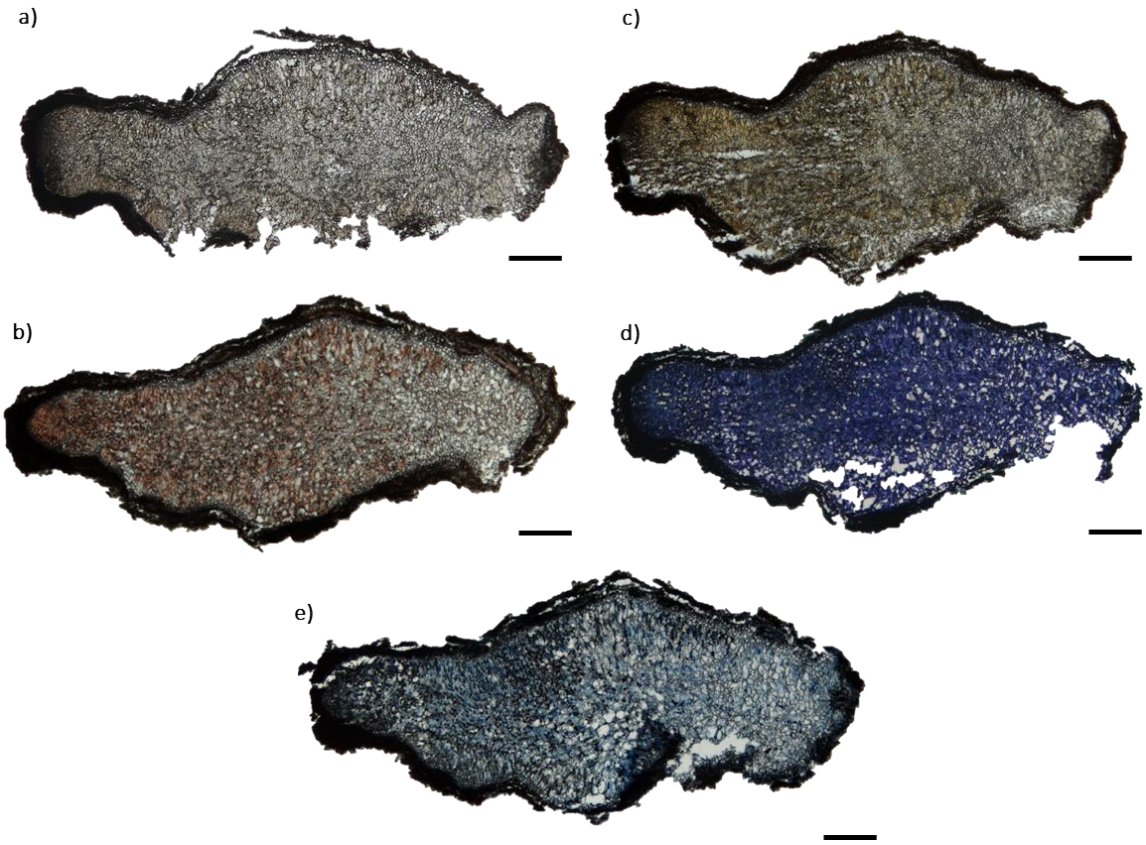


Figure 11 – Longitudinal sections obtained with freezing microtome of *A. longifolia* young plants nodules from unburnt zone with different staining procedures: a) no staining, b) Sudan red, c) Lugol, d) toluidine blue and e) lactophenol and blue cotton solution. Images can be from different nodules. The images were obtained with Zeiss Lumar V12 Stereoscope. Scale bar = 1.5 mm.

Figure 12 – Longitudinal sections obtained with freezing microtome of *A. longifolia* young plants nodules from burnt zone with different staining procedures: a) no staining, b) Sudan red, c) Lugol, d) toluidine blue and e) lactophenol and blue cotton solution. Images can be from different nodules. The images were obtained with Zeiss Lumar V12 Stereoscope. Scale bar = 1.5 mm.

4.3 Bacteria collection, characterization and fingerprinting

A total of 152 isolates were obtained from *A. longifolia* young plants nodules, 92 from unburnt zone and 60 from burnt zone (Fig. 13).

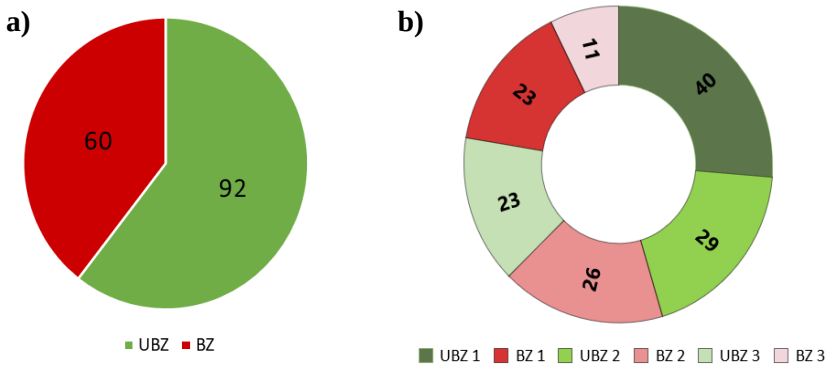


Figure 13 – Schematic diagram of the total number of nodules' isolates a) in unburnt and burnt zones and b) in the six sampled sites, three unburnt zones and three burnt zones. Unburnt zones represented by UBZ and burnt zones represented by BZ zones.

Regarding the biochemical tests applied to each isolate, for Gram's test it was observed that Gram-negative isolates were present in both unburnt and burnt zones (82 and 55, respectively). In what concerns the catalase test, there were more catalase positive isolates obtained from both unburnt and burnt zones (81 and 48, respectively) comparing to catalase negative ones. For both zones, oxidase test response was positive for all isolates (Fig. 14).

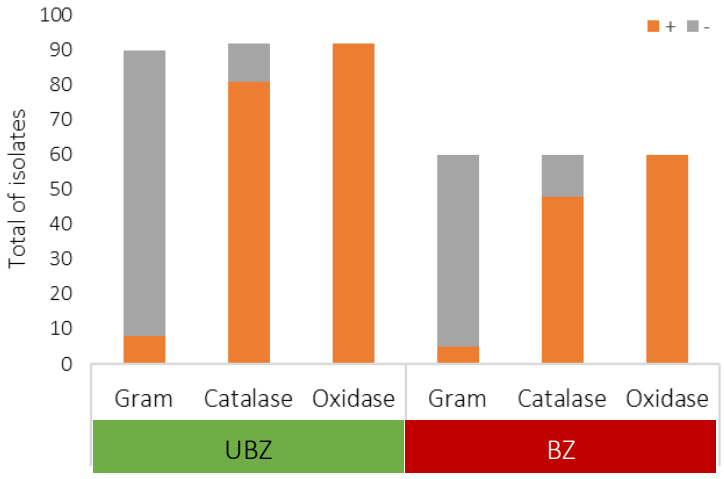


Figure 14 – Total of nodules' isolates distributed according to its response to each biochemical test (Gram, catalase and oxidase) in both sampled zones (unburnt zones, UBZ, and burnt zones, BZ). Positive results are represented with (+) and negative results are represented with (-).

Colonies morphology was diverse. In terms of pigmentation, while all the colonies present in burnt zone were white, in unburnt zone other colors were present (Fig. 15b). It is important to mention that its aspect also varied between creamy, mucous or milky. Regarding the margins, there was a colony with a filamentous margin in burnt zone (Fig. 15c); in terms of size and elevation there were no differences (Fig. 15a and d). All the OTUs (Operational Taxonomic Units) obtained formed visible colonies between 2- and 10- days growth on YMA plates.

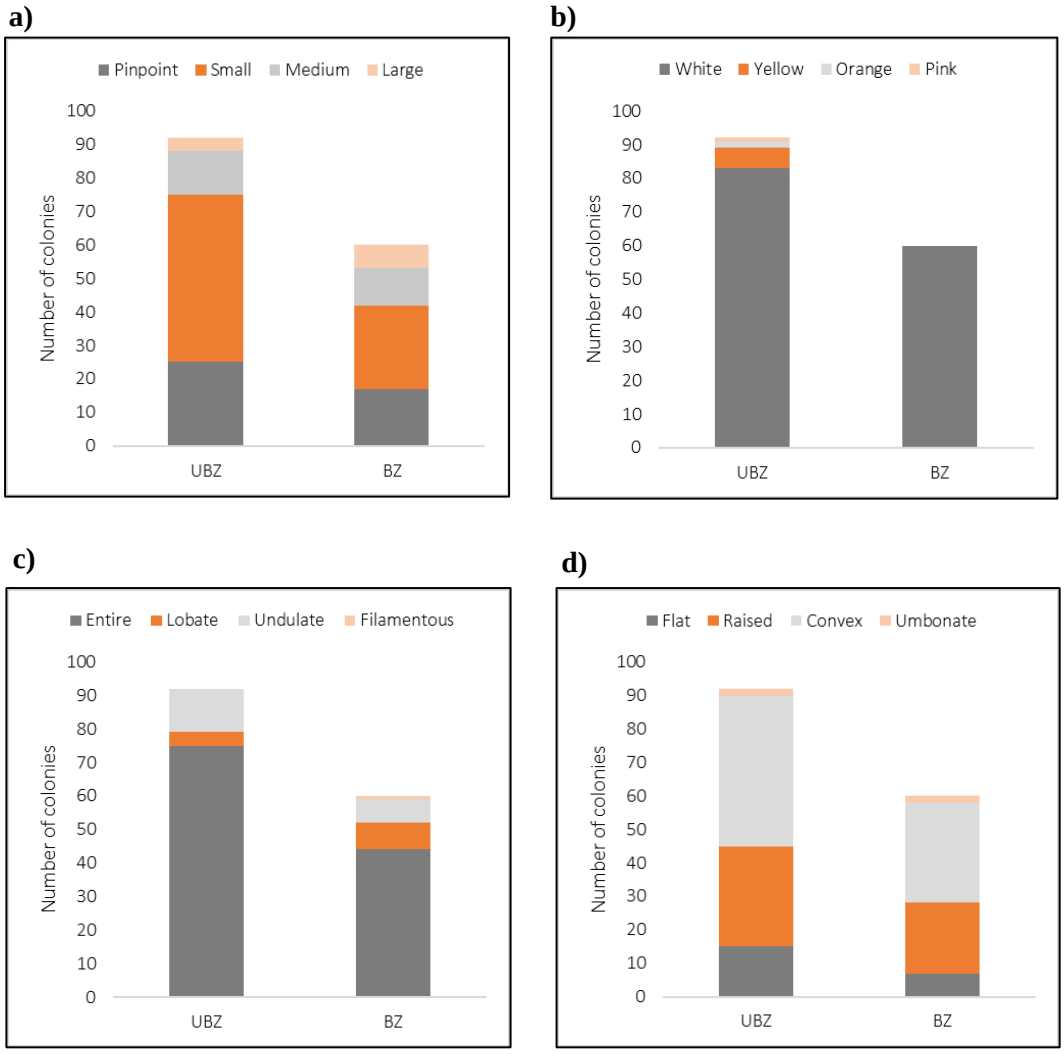


Figure 15 – Total number of nodules' isolates of unburnt (UBZ) and burnt (BZ) zones distributed according to its colony characteristics: a) Size – Pinpoint, Small, Medium and Large; b) Pigmentation – White, Yellow, Orange and Pink; c) Margin – Entire, Lobate, Undulate and Filamentous and d) Elevation – Flat, Raised, Convex and Umbonate.

After phenotypic analysis, genomic fingerprinting based on M13 was performed and the results are presented in the dendrogram. As shown in Fig. 16, in unburnt zone, the 92 isolates were organized in 22 clusters; in burnt zone, the 60 isolates were organized in 13 clusters (Fig. 17). Some of the isolates were clustered independently, forming a group with a unique representative.

There was a higher genera diversity of bacteria associated with *A. longifolia* in unburnt zones. Also, the dendrogram for all the 152 isolates, 92 from unburnt zone and 60 from burnt zone, showed that these isolates were clustered all together and there was no segregation between isolates from unburnt zone and burnt zone (Appendix 6).

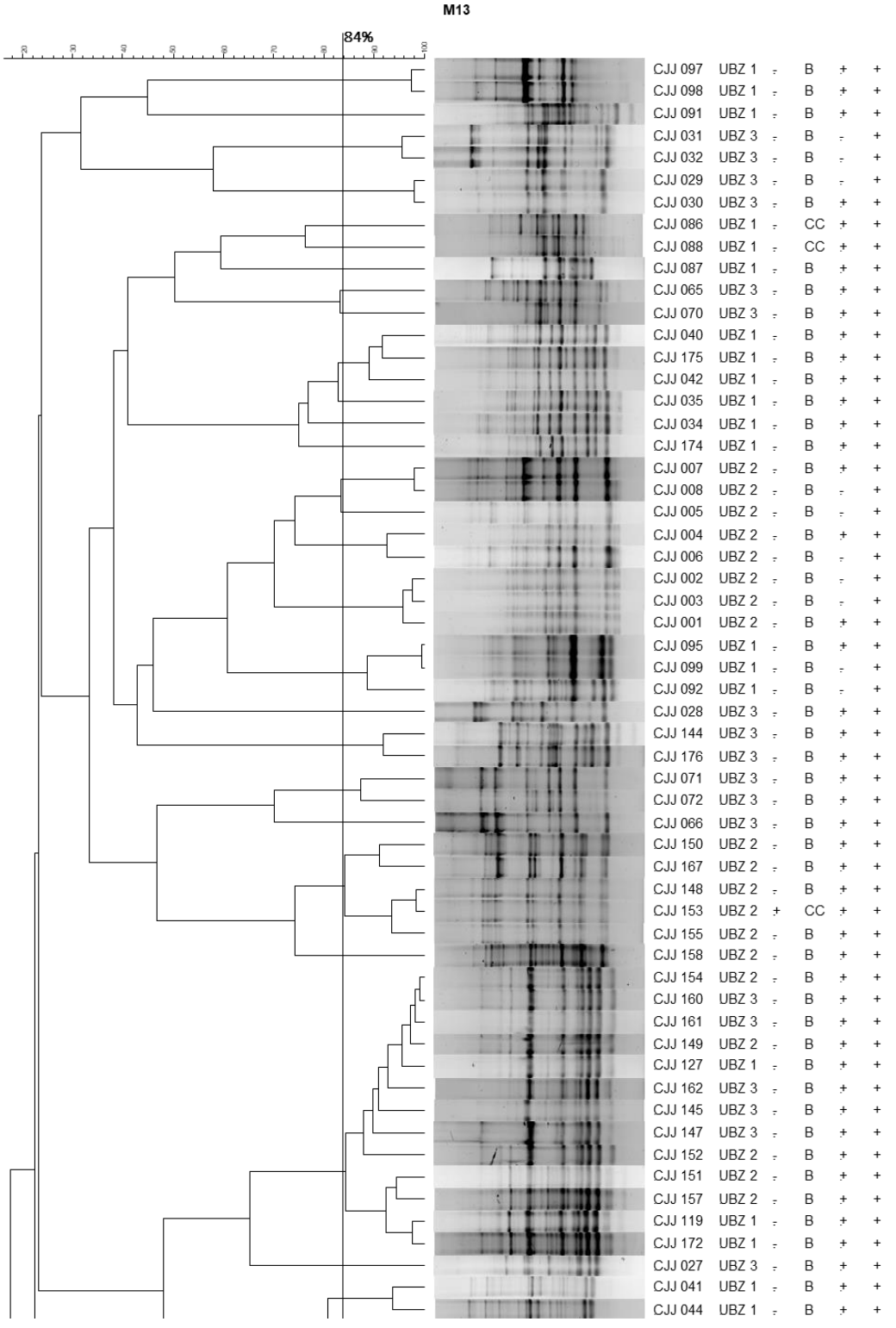
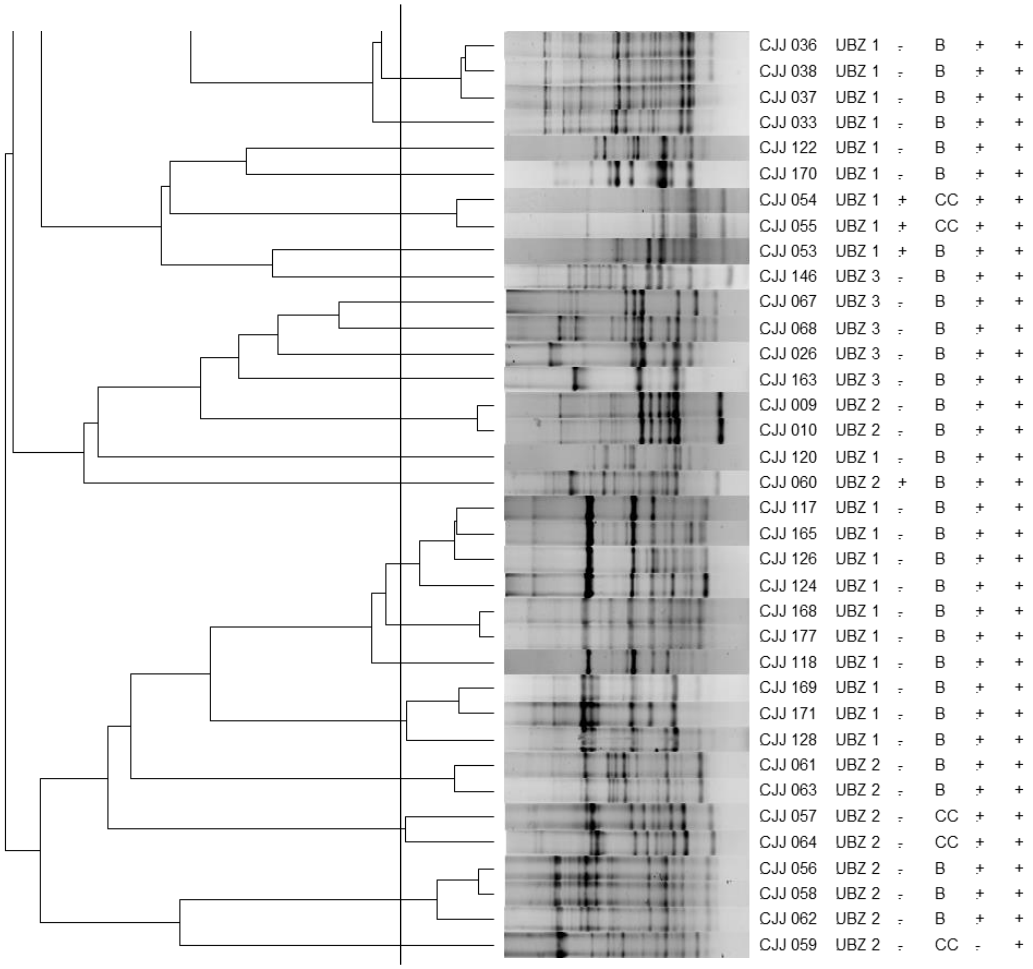


Figure 16 - Dendrogram based on cluster analysis of fingerprinting PCR products, using the unweighted pair-group method with arithmetic mean algorithm (UPGMA) and the Pearson correlation coefficient, of the isolates from nodules of *A. longifolia* young plants. UBZ, isolates from unburnt zones (1,2 and 3). The figure continues is the next page.


CJJ 036 UBZ 1 - B + +
CJJ 038 UBZ 1 - B + +
CJJ 037 UBZ 1 - B + +
CJJ 033 UBZ 1 - B + +
CJJ 122 UBZ 1 - B + +
CJJ 170 UBZ 1 - B + +
CJJ 054 UBZ 1 + CC + +
CJJ 055 UBZ 1 + CC + +
CJJ 053 UBZ 1 + B + +
CJJ 146 UBZ 3 - B + +
CJJ 067 UBZ 3 - B + +
CJJ 068 UBZ 3 - B + +
CJJ 026 UBZ 3 - B + +
CJJ 163 UBZ 3 - B + +
CJJ 009 UBZ 2 - B + +
CJJ 010 UBZ 2 - B + +
CJJ 120 UBZ 1 - B + +
CJJ 060 UBZ 2 + B + +
CJJ 117 UBZ 1 - B + +
CJJ 165 UBZ 1 - B + +
CJJ 126 UBZ 1 - B + +
CJJ 124 UBZ 1 - B + +
CJJ 168 UBZ 1 - B + +
CJJ 177 UBZ 1 - B + +
CJJ 118 UBZ 1 - B + +
CJJ 169 UBZ 1 - B + +
CJJ 171 UBZ 1 - B + +
CJJ 128 UBZ 1 - B + +
CJJ 061 UBZ 2 - B + +
CJJ 063 UBZ 2 - B + +
CJJ 057 UBZ 2 - CC + +
CJJ 064 UBZ 2 - CC + +
CJJ 056 UBZ 2 - B + +
CJJ 058 UBZ 2 - B + +
CJJ 062 UBZ 2 - B + +
CJJ 059 UBZ 2 - CC - +

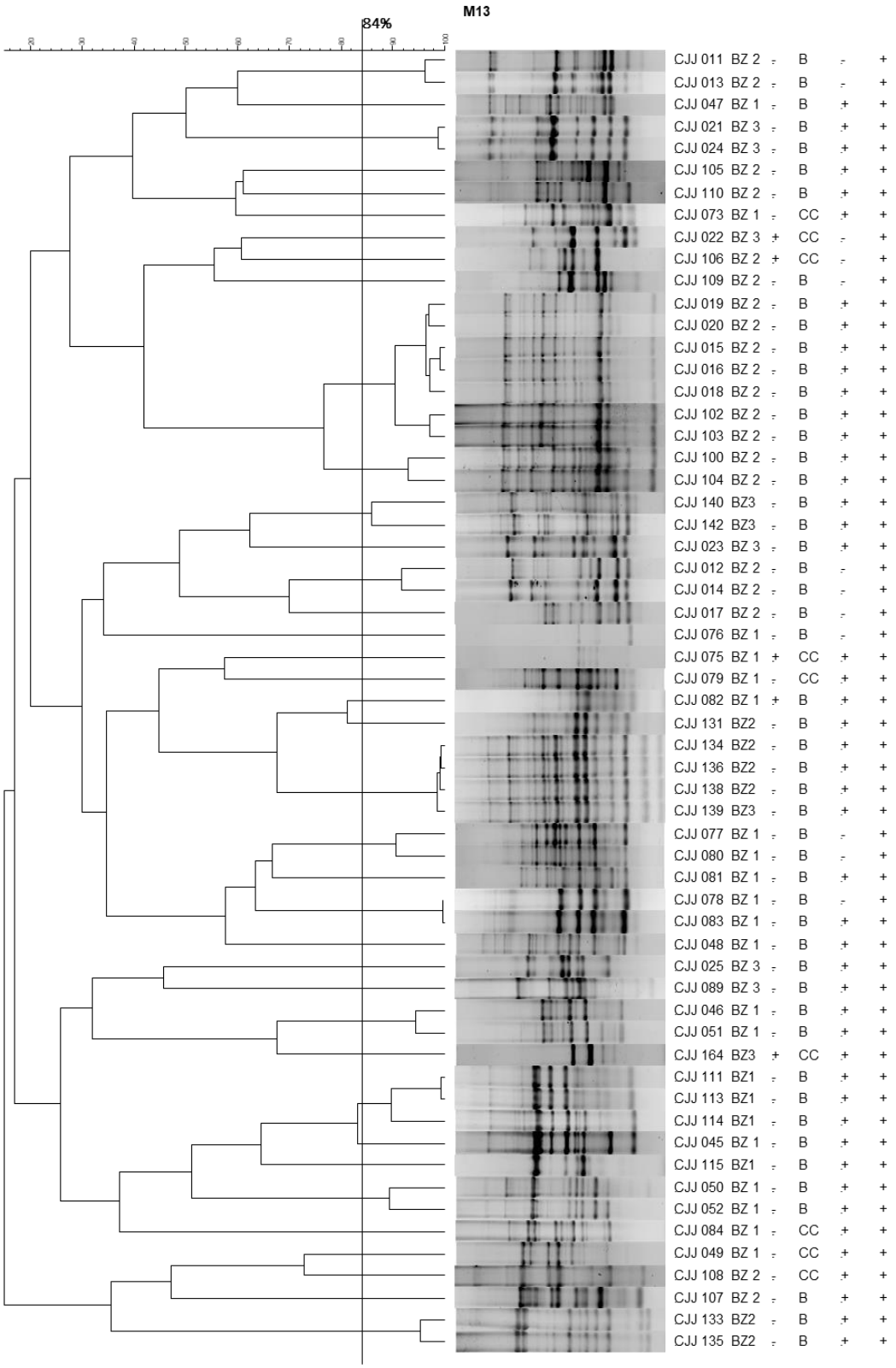


Figure 17 - Dendrogram based on cluster analysis of fingerprinting PCR products, using the unweighted pair-group method with arithmetic mean algorithm (UPGMA) and the Pearson correlation coefficient, of the isolates from nodules of *A. longifolia* young plants. BZ, isolates from burnt zones (1,2 and 3).

4.4 Alignments

BLAST analysis following sequencing allowed the preliminarily identification of 45 isolates. The percentage of pairwise identity was higher than 90 % for all isolates analyzed. Some isolates were identified up to species level (Table 5).

Table 5 – BLAST analysis of the sequences obtained from bacterial isolation from unburnt and burnt zones. * The description result showed is the one that present the higher percentage of pairwise identity; two or three results are shown only when equal percentage of identity were obtained for the same sequence. The table ends in the following page.

Isolate ID	Description*	% Pairwise Identity	GenBank Access Number
3	*Bradyrhizobium sp.*	100 %	MH612954
8	*Bradyrhizobium sp.*	100 %	Z94815
10	*Bradyrhizobium cytisi*	98.1 %	MK370569
13	*Bradyrhizobium pachyrhizi*	100%	KP769443
17	*Bradyrhizobium cytisi*	99.3 %	MK370569
20	*Bradyrhizobium sp.*	99.5 %	DQ202229
24	*Bradyrhizobium cytisi*	100 %	MK370569
25	*Paraburkholderia phytofirmans*	100 %	NR_102845
26	*Bradyrhizobium ganzhouense*	99.8 %	NR_133706
	Bradyrhizobium rifense	99.8 %	NR_116361
27	*Althererythrobacter sp.*	99.4 %	MG450544
28	*Bradyrhizobium cytisi*	100 %	MK370569
30	*Bradyrhizobium cytisi*	100 %	MG588695
32	*Bradyrhizobium sp.*	100 %	MH698652
38	*Pseudomonas moorei*	99.3 %	KF704108
44	*Pseudomonas sp.*	99.6 %	LN995693
46	*Burkholderia sp.*	99.8 %	KF922657
50	*Burkholderia sp.*	94.2 %	KR154612
53	*Uncultured bacterium*	99.5 %	HM330647
	Nocardioides oleivorans	99.4 %	KY753200
54	*Micrococcus luteus*	99.8 %	NR_05062
	Micrococcus aloeverae	99.8 %	MG966301
	Micrococcus yunnanensis	99.8 %	LN774334
56	*Rhizobium rhizogenes*	100 %	EU420078
60	*Paraburkholderia caledonica*	99.8 %	NR_114117
63	*Bradyrhizobium sp.*	100 %	MH698652
65	*Bradyrhizobium cytisi*	99.8 %	MK370569
67	*Paenibacillus glucanolyticus*	99.7 %	R_113748
72	*Bradyrhizobium cytisi*	98.8 %	MK370569

75	*Micrococcus sp.*	99.4 %	KF054881
76	*Bradyrhizobium cytisi*	99.8 %	MK370569
80	*Bradyrhizobium cytisi*	99.6 %	MK370569
81	*Bradyrhizobium sp.*	99.2 %	KX838338
83	*Bradyrhizobium sp.*	99.8 %	MH698652
86	*Moraxella osloensis*	99.9 %	MK51189
	Enhydrobacter sp.	99.9 %	MH703459
87	*Burkholderia sp.*	99.3 %	KY681989
97	*Caballeronia udeis*	94.7 %	MK302235
	Caballeronia sp.	94.7 %	MH018897
	Uncultured Burkholderiales	94.7 %	LC213287
99	*Bradyrhizobium cytisi*	99.8 %	MK370569
105	*Paraburkholderia sp.*	99.8 %	MK373510
107	*Paraburkholderia sp*	100 %	MK373510
109	*Burkholderia fungorum*	96.7 %	LC104284
	Uncultured Burkholderia	96.7 %	JN590614
115	*Paraburkholderia sp.*	92.1 %	MK373650
119	*Burkholderia sp.*	99.6 %	MK574764
120	*Pseudomonas helmanticensis*	99.9 %	MG269630
	Pseudomonas fluorescens	99.9 %	MF618323
	Pseudomonas baetica	99.9 %	KC90260
122	*Pseudomonas sp.*	99.8 %	MK559942
124	*Duganella sp.*	98.2 %	JF904873
133	*Bradyrhizobium canariense*	99.2 %	MG588599
139	Uncultured bacterium	98.1 %	JF200701
	Pseudomonas sp.	98.1 %	MK610664
	Pseudomonas fluorescens	98.1 %	MK355572
	Pseudomonas koreensis	98.1 %	MK026822
142	*Bradyrhizobium cytisi*	99.8 %	MK370569
144	*Bradyrhizobium cytisi*	99.9 %	MG588679
146	*Pseudomonas sp.*	100 %	MK610664
	Pseudomonas fluorescens	100 %	MK355572
	Pseudomonas koreensis	100 %	MK026822
	Pseudomonas moraviensis	100 %	MK240436
149	*Paraburkholderia sedimicola*	99.7 %	MK574764
164	*Micrococcus yunnanensis*	99.1 %	KP406728
171	*Pseudomonas helmanticensis*	100 %	MG269630
	Pseudomonas fluorescens	100 %	MF618323
	Pseudomonas baetica	100%	KC90260
174	*Bradyrhizobium ganzhouense*	99.7 %	NR_133706
	Bradyrhizobium rifense	99.7 %	NR_116361

Considering the diversity of bacteria, it is observed that there was more diversity in unburnt zone, in terms of phylum and genera (Fig. 18 and Fig. 19). It is observed that considering the 152 isolates obtained, the 92 isolates from unburnt zone were distributed in 5 different classes: Alphaproteobacteria (39.1 %), Betaproteobacteria (26.1 %) and Gammaproteobacteria (16.3 %) from phylum proteobacteria; Actinobacteria (5.4 %) from phylum actinobacteria and Bacilli (1.1 %) from phylum firmicutes. There were 12% of the isolates that remained unknown; the 60 isolates from burnt zone were distributed in 4 different classes: Alphaproteobacteria (45 %), Betaproteobacteria (15 %) and Gammaproteobacteria (10 %) from phylum proteobacteria; Actinobacteria (8.3 %) from phylum actinobacteria. Also, there were 26.1 % of the isolates still unknown (Fig. 18).

The genus *Bradyrhizobium* is the most prevalent, followed by the genus *Pseudomonas* and the genus *Parabulkholderia* in both zones. In burnt zone, the genera *Rhizobium*, *Althererythrobacter*, *Caballeronia*, *Duganella*, *Moraxella*, *Paracoccus* and *Paenibacillus* are not present, despite its presence in unburnt zone (Fig. 19).

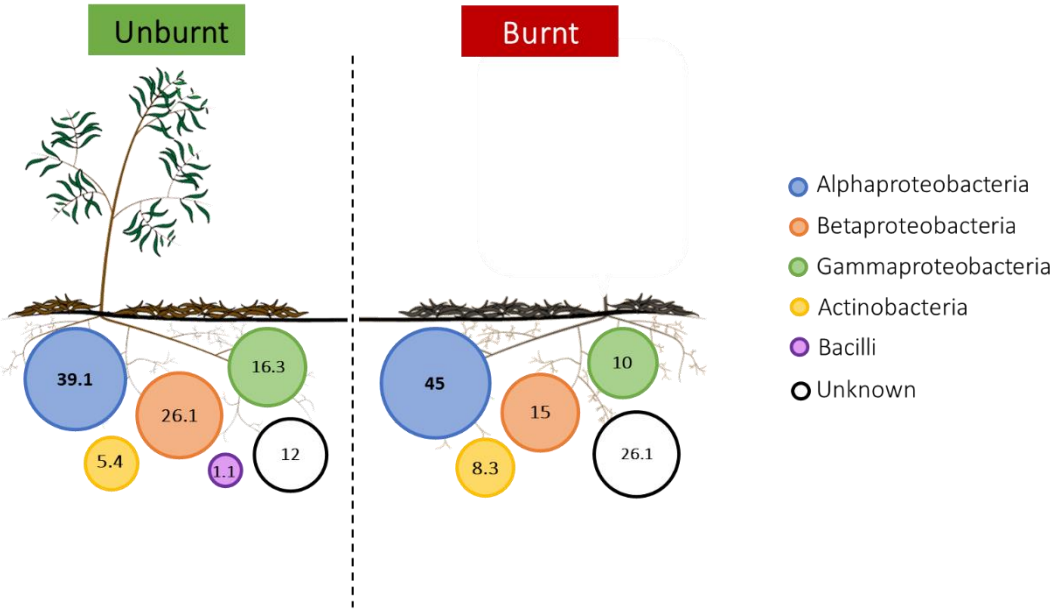


Figure 18 – Percentage of the distribution of the different classes of bacteria isolates from *A. longifolia* young plants nodules, present in both unburnt and burnt zones.

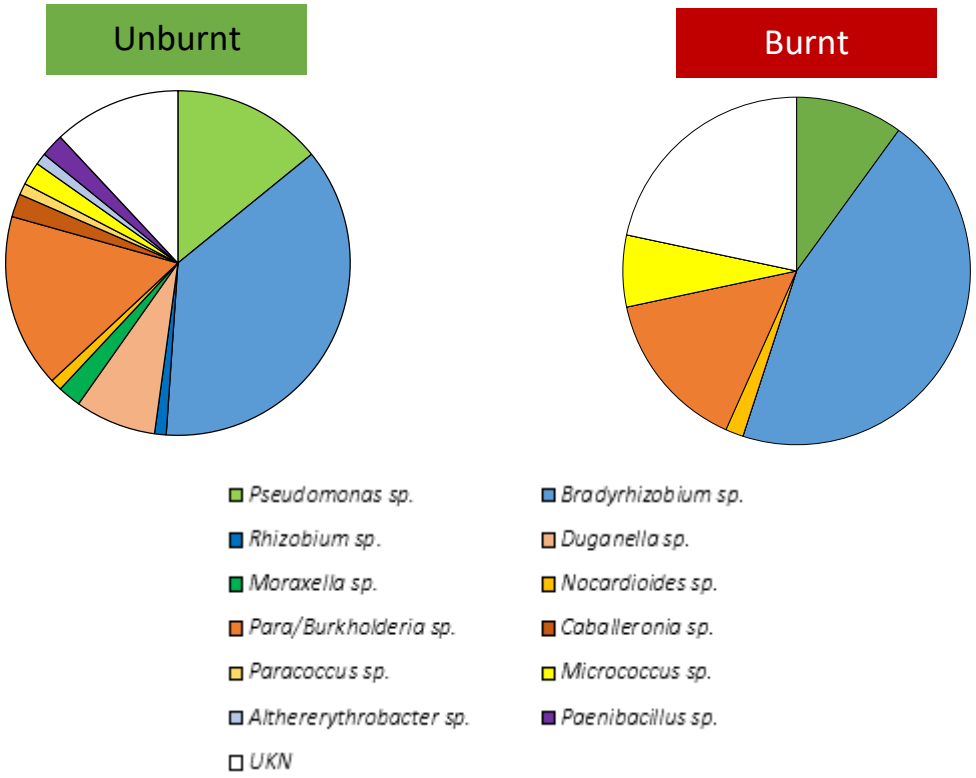


Figure 19 – Total of bacterial isolates from *A. longifolia* young plants nodules classified by genera, present in both unburnt and burnt zones.

4.5 Diversity and Evenness indexes

As shown by Shannon-Wiener diversity index, there was more isolated bacteria diversity in unburnt zone (H' = 1.0) than in burnt zone (H' = 0.74); for Pielou evenness index, it was observed that in both zones there were isolates that are dominant (J' = 0.75 for unburnt zone and 0.67 for burnt zone); for Simpson diversity index, was also observed a high diversity for unburnt zone (D' = 0.97) and for burnt zone (D' = 0.98) (Table 6).

Table 6 – Shannon-Wiener and Simpson diversity indexes and Pielou evenness index for unburnt (UBZ) and burnt (BZ) zones from bacterial isolates from *A. longifolia* young plants nodules.

	Shannon-Wiener Diversity Index	Pielou Evenness Index	Simpson Diversity Index
UBZ	H' = 1.0	J' = 0.75	D' = 0.97
BZ	H' = 0.74	J' = 0.67	D' = 0.98

4.6 Next Generation Sequencing (NGS)

Regarding the Next Generation Sequencing analysis, the most represented genus was *Bradyrhizobium* in both zones, followed by *Parabulkholderia*. *Massilia* genus was the third group more represented in unburnt zone, while in burnt zone was *Caballeronia*. It is also important to mention that different genera were present in both zones, however they were less represented (Table 7).

Table 7 – Bacteria genera represented in *A. longifolia* young plants nodules from unburnt and burnt zones obtained through Next Generation Sequencing.

Unburnt		**Burnt**	
Genera	**Reads obtained**	**Genera**	**Reads obtained**
Bradyrhizobium	405758	*Bradyrhizobium*	330383
Paraburkholderia	18914	*Paraburkholderia*	11471
Massilia	10205	*Caballeronia*	3674
Caballeronia	4714	*Rhodopseudomonas*	1172
Tatumella	1448	*Tatumella*	1134
Rhodopseudomonas	1375	*Massilia*	1097
Tardiphaga	1005	*Stanieria*	750
Francisella	755	*Tardiphaga*	602
Stanieria	664	*Bacillus*	585
Kosakonia	537	*Sneathiella*	437
Burkholderia	337	*Oscillatoria*	382
Phenylobacterium	310	*Aerosakkonema*	275
Total of reads classified	783333	**Total of reads classified**	646529

Analyzing the OTUs until species level, it was observed a higher intraspecific diversity inside *Bradyrhizobium* genus and the species more represented was *Bradyrhizobium cytisi*, as well as, *Tatumella terrea* was the less represented in both zones (Fig. 20 and 21). In unburnt zone, *Bradyrhizobium* is followed by three genera from Betaproteobacteria: *Paraburkholderia*, and *Caballeronia*, as single species genera represented by *Paraburkholderia caledonica* and *Caballeronia concitans* respectively; and *Massilia*, with two species (*Massilia revitalea* and *Massilia putida*) (Fig. 20).

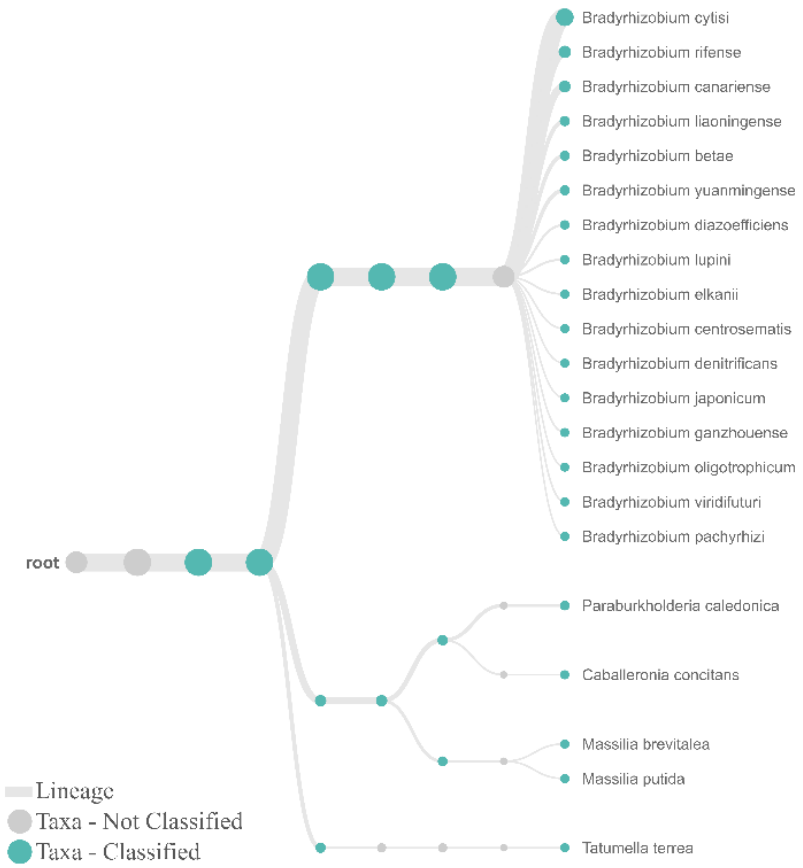


Figure 20 – Taxonomic trees representing only the first 20 species obtained from *A. longifolia* young plants nodules in unburnt zone. The thickness of the branches is proportional to its abundance and the colored points represent the OTUs (Operational taxonomic units) that were classified in each taxonomic level.

In burnt zone, *Rhodopseudomonas palustris* is present and *Massilia* genus is also present but in a lower frequency, not being represented amongst the first 20 species shown in taxonomic tree. In what concerns Betaproteobacteria diversity, *Paraburkholderia* is represented by three species (*P. phytofirmans, P. tuberum* and *P. sacchari*) and *Caballeronia* has two species represented (*C. jiangsuensis* and *C. megalochromosomata*) (Fig. 21).

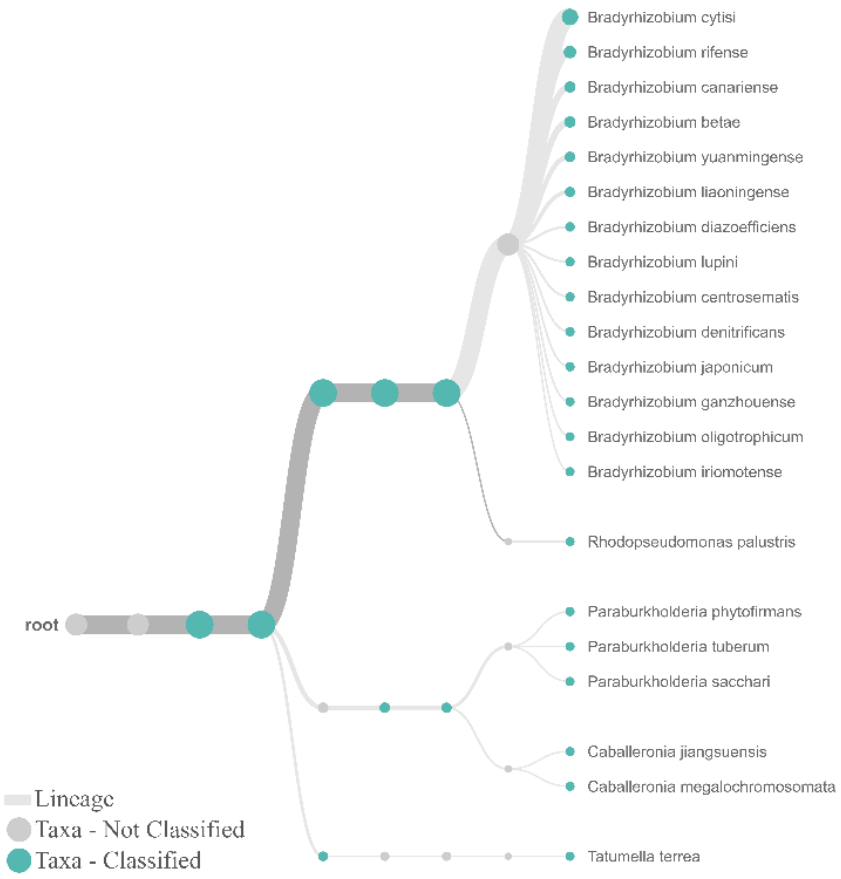


Figure 21 – Taxonomic trees representing only the first 20 species obtained from *A. longifolia* young plants nodules in burnt zone. The thickness of the branches is proportional to its abundance and the colored points represent the OTUs (Operational taxonomic units) that were classified in each taxonomic level.

4.7 Isotopic analysis

Isotopic analysis showed similar results in nodules from both zones, however N and C percentages were slightly higher in burnt zone compared to unburnt zone (6.3 and 5.3 of % N and 42.3 and 36.5 of % C, respectively). In leaves, the $\delta^{15}N$ was closer to zero in both unburnt zone (-1.0 ‰) and burnt zone (0.8 ‰) and the other results are similar (Table 8).

Table 8 – Isotopic analysis of nitrogen and carbon in *A. longifolia* young plants leaves and nodules from unburnt (UBZ) and burnt (BZ) zones. The natural relative abundance of N and C is represented by $\delta^{15}N$ and $\delta^{13}C$, respectively, and the percentage of these elements in leaves and nodules by % N and % C. The ratio C/N is also shown. No statistically significant differences, according to T-test with an $\alpha = 0.05$.

	Leaves					Nodules				
	$\delta^{15}N$	$\delta^{13}C$	%N	%C	C/N	$\delta^{15}N$	$\delta^{13}C$	%N	%C	C/N
UBZ	-1.0	-30.2	2.4	44.3	19.1	7.9	-29.5	5.3	36.5	7.0
BZ	0.8	-29.5	3.3	43.5	15.2	7.4	-28.8	6.3	42.3	7.0

5. Discussion

Biological invasions can be studied from different points of view and encompass such a variety of areas in order to correlate data, understand species behavior and make conscious decisions to implement control measures. *A. longifolia* is an interesting species for these studies, and can actually be proposed as a model species, due to all its impacts and incredible adaptations, as well, the fact that is considered a global invader.

After fire, *Acacia sp.* seed germination is stimulated due to a clear adaptation to fire regimes (Weiss, 1984), thus, the study of young plants invasive behavior under fire regimes, in particularly the establishment of symbiotic interactions with bacteria communities is justified, given the lack of information in this level. On the other hand, finding the adequate symbiotic partners can determine the success or the decline of the invader. Moreover, after fire, it is fundamental to clarify the relation's belowground between microbial communities and the invader *Acacia sp.* since the "bacteriome" inside root nodules could be different.

Nodules: what is happening?

In this study, burnt soil analysis revealed highest amount of ammonium (NH_4^+) and nitrate (NO_3), which is in accordance with the inorganic forms of nitrogen originated after fire, ammonium forming as a direct product of the combustion while nitrate is biochemically formed weeks or months later through nitrification (Covington and Sackett, 1992). Also, denitrification and nitrification processes that occur in soils can contribute to this large amount of these mineral forms of nitrogen in soils, both unburnt and burnt (Godfrey and Glass, 2011). If so, why all this investment in nodule formation by *A. longifolia*?

A. longifolia' nodules in what concerns anatomy, are classified as indeterminate according to the classification established for legumes by Franssen *et al*. (1992) and Maunoury *et al*. (2008), which means that an elongation process of the nodule meristem must occur for it to become cylindrical and with different zones. Those nodules had different shapes: cylindrical, cylindrical and bilobate and cylindrical and trilobate. Nodules with spherical forms, as previously reported by Lopez-Lara *et al*. (1993) were also observed. Previous studies described that leguminous plants could downregulate their nodule number, size and nitrogen fixation activity when enough nitrogen is available in the soil (Carroll *et al.*, 1985; Streeter and Wong, 1988).

In fact, we may hypothesize that these *A. longifolia* young plants may respond to fire events, showing a different behavior in a "new" environment after fire, which may be particularly relevant for fitness. The acquisition of symbiotic partners allows nitrogen fixation, which allows

A. longifolia to overcome other species; for this reason, nodulation seems to be a good process to allocate energy in and this can be seen in the higher number of nodules after fire.

A higher value of total nitrogen in burnt soils is a result that is not in accordance with previous findings by Blair *et al.* (1997) that showed a decrease in the availability of total nitrogen immediately after a fire. This discrepancy might be related to the fact that plants were collected one year after the fire, and this period can be enough for biochemical alterations in nitrogen (and carbon). Furthermore, there are also legacy effects (especially in nutrient cycles) let by *Acacia spp.* in soils, another reason why it is considered an invasive transformer (Richardson *et al.*, 2000).

Moreover, Vasse *et al.* (1990) described that, during the elongation process of the nodule meristematic zone, several histological zones are developed. Active nitrogen-fixing nodules become red in nitrogen-fixing zone which corresponds to the zone where bacteria go through a differentiation process until bacteroid form (Dupont *et al*., 2012) and this is linked with the presence of leghemoglobin, responsible for microaerophilic environment inside the nodule that allows nitrogenase activity. In our study, nodules in different maturation stages were observed in *A. longifolia* young plants, with the same age.

In addition, isotopic analysis directly performed in the nodules revealed the absence of nitrogen fixation activity inside them once, according to Godfrey and Glass (2011), values of $\delta^{15}N$ between -2 and 2 ‰ indicate BNF and the values obtained were out of this range; however, values of $\delta^{15}N$ obtained from *A. longifolia* leaves, were included in that range, so nitrogen provided from symbiotic fixation is a source of nitrogen used by plant. The observed difference between nodules and leaf material may be associated with the fact that the material of the nodule suffered additional fractionation during post-nitrogen fixation. All these observations point out to nodulation being a dynamic process, as well as nitrogen fixation, where some nodules are starting its development, others are becoming senescent, being present in roots, nodules with different sizes (Appendix 7). This highlights the importance of nodules as a remarkable part of the plant, along with leaves or roots. This ultimately can justify the same development behavior.

Besides this agreement between leaf isotopic analysis and nodules morphological observation, future isotopic analysis should be done in carefully way, once nodules material may present high quantities of plant material that can interfere with the isotopic signal associated with atmospheric nitrogen fixation. A methodological option could pass by peeling the nodules before being processed for these studies in order to have a more certain analysis.

In this sense, what can explain this possible “dynamic” nitrogen fixation?

Interestingly, for grain legumes, nitrogen fixation is optimal during 4-5 weeks after infection and its downtime triggers senescence. Once senescence starts, a fast decrease in bacteroid fixation capacity is detectable in nitrogen-fixation zone inside nodule, where it is described to start at (Bethlenfalvay and Phillips, 1977; Lawn and Brun, 1977), turning from pink to green (Schumpp and Deakin, 2010).

Additionally, this can be corroborated with Hansen *et al.* (1987) studies of nitrogenase activity in nodules from two species of *Acacia* in its native range, where this enzyme is described to have a seasonal profile. This study confirms that, in first year seedlings after a fire, where nodules are formed during the first 4 months corresponding to the late Autumn and during Winter, and in August (end of Winter and beginning of Spring), nitrogenase has its peak of activity, followed by a fast decrease of almost 75 %. After that, until the end of Spring, during Summer and beginning of Autumn, its activity is very residual. If it is hypothesized that the same seasonal activity profile occurs in *Acacia sp.* invasive range (e.g. Portugal), under these conditions (with a fire occurred in October), nitrogenase activity reaches its peak in April, remaining active during 4-5 weeks and starting to decrease, reason why in October when nodules were collected, only a few remained intact and functional.

Furthermore, and besides the fact that the senescence process is nodule type dependent, it is reported by Werner *et al.* (2010) that, under water stress conditions, as occurs after fire (Neary *et al.*, 1999), *A. longifolia* revealed as a highly sensitive plant to drought, leading to nitrogen-uptake efficiency largely decreased, which is directly related to nitrogenase activity too.

For this reason, further studies should rely on understanding and confirm this seasonal pattern of nitrogenase regarding *A. longifolia* native range in order to understand the impact in nodule tissues and its behavior under water stress, along with histological characterization.

"Bacteriome": does fire play a role?

Besides the necessity of nitrogenase to fix N_2, for BNF, nitrogen-fixing bacteria are essential. Whilst the higher number of nodules was observed in burnt zone, the diversity of bacteria obtained through fingerprinting analysis was higher in unburnt zone, independently of the quantity of isolates obtained. This is corroborated by a higher value of Shannon-Wiener diversity index for unburnt zone. However, an important aspect that should be included in further studies is the intensity of fire that could give information about soil microbiome losses associated with temperature, for example. This could be a primarily hypothesis for the lower diversity found in burnt zones. Despite this, it is important to highlight that, apart from the lower bacterial diversity observed in burnt zone, most of the genera present were already described as nitrogen fixers or with *nif* genes present (Franche, 2009). For this reason, fire seems to be a relevant factor for symbiosis.

Our study was conducted to understand "who" takes part of this "bacteriome", since accordingly to Martínez-Hidalgo and Hirsch (2017), nitrogen-fixing bacteria do not live alone inside nodules.

As expected, both fingerprinting and NGS approaches, showed that there are a large representation of Proteobacteria in *A. longifolia* young plant nodules. In addition, Pielou evenness index showed that there were present, in both unburnt and burnt zones, species that could have

certain dominance. As expected, the common *Bradyrhizobium* genus, included in rhizobia, was the most abundant in both unburnt and burnt "bacteriome" inside nodules, revealing its dominance. Furthermore, there was also present an intraspecific diversity with *Bradyrhizobium cytisi* as the most abundant species. Following this genus, P*araburkholderia spp.* was also a highly represented nitrogen-fixing inducer isolated. Besides *Bradyrhizobium spp.*, amongst Alphaproteobacteria, one of the most curious findings is the absence of *Rhizobium*, *Mesorhizobium* and *Ensifer* genera in great abundance, as described in other legumes (Franche, 2009) and inclusively in *Acacia spp*. (LaFay and Burdon, 2001; Marsudi *et al.*, 1999; Nick *et al.*, 1999a and b).

Moreover, we found *Pseudomonas sp.* and *Paenibacillus sp.*, two examples represented in isolates obtained in our study, which accordingly to Martínez-Hidalgo and Hirsch (2017) are non-rhizobial nodule inducing bacterial endophytes (NRE), able to invade the infection threat when co-inoculated with rhizobia.

Massilia genus was also present in both zones as shown by NGS, but almost 10 times higher in unburnt zone. Ofek *et al.* (2012) described *Massilia* genus (Oxalobacteraceae like *Duganella*) as an emerging genus detected inside roots and present in rhizosphere of many plant species with an ecological meaningful interest, but also a dynamic behavior sensitive to environment. In fact, *Massilia spp.* were described to be associated with some agricultural soils (Grönemeyer *et al*., 2012). Following this reasoning, it is curious to highlight that this genus is present in the sampled zones closer to agricultural fields (Appendix 1).

The question that arises is: how such a diverse "bacteriome" in *A. longifolia* young plant nodules?

On one hand, with so many microorganisms present in soils, horizontal/lateral gene transfer (H/LGT) is one of the easiest environmental process especially when the most abundant bacteria found were phylogenetically close (Proteobacteria) (Sullivan and Ronson, 1998; Rogel *et al.*, 2001; Ramsay *et al.*, 2006, 2009). Oddly enough, a particularity of these isolates obtained is that most of the colonies observed were mucous.

As shown by Hirsch (1999), exopolysaccharides (EPS), responsible for this mucus, can be determinant in signal exchange between *Acacia sp.* and rhizobia during infection process. It can be hypothesized that other bacteria than rhizobia can acquire these genes through HGT and secrete EPS to "mimic" rhizobia (Fig. 22), winning a place inside nodule and "cheating" *A. longifolia*. In fact, there is a high probability for the occurrence of evolutionary mechanisms *via* a gain of function (Buckling *et al*., 2009; Brockhurst *et al*., 2011) and the ability to behave as a symbiont or NRE, because besides selective pressures such as plant immunity and nutrition (Guan *et al.*, 2013), it can improve fitness, once is a mutualism and certain gains are involved (such as nutrition or just protection).

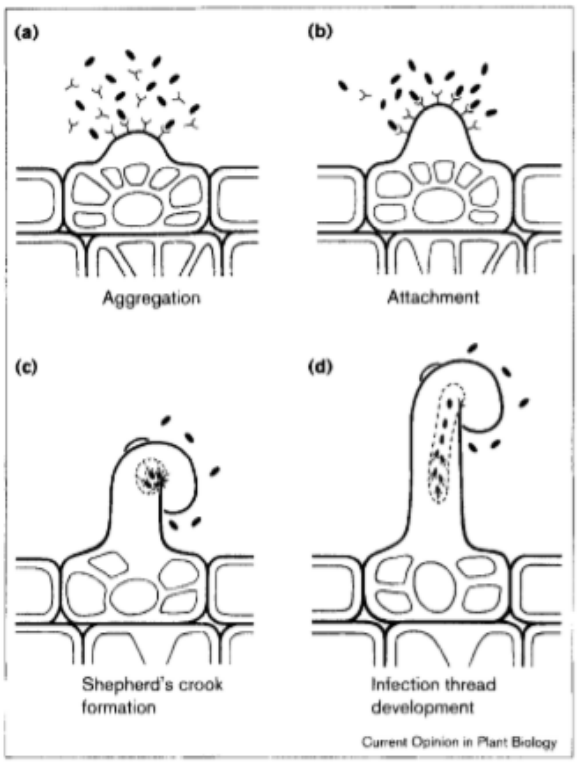


Figure 22 - Diagram by Hirsch (1999) of how lectin is presumed to function. (a) Lectins (Y) are localized and secreted from the emerging root hair facilitating rhizobial aggregation. (b) Rhizobia are attached to the root hair. (c) Root hair curls around the rhizobia and an infection thread forms at the "hyaline spot". Rhizobia are bound by lectin within the nascent thread. (d) The Infection thread extends, and the root hair elongates. Rhizobia multiply within the thread.

Genes involved in signal exchange and nodulation, like nod factors, responsible for triggering plant developmental program easily that is required for root hair infection, leading to nodule formation (Gough and Cullimore, 2011) and allowing infection, could be studied in order to understand its specificity, once its location is on symbiotic plasmids or highly mobile "symbiotic islands", which can be transferred easily between different bacterial species, and even genera (Ding and Hynes, 2009).

On the other hand, such diverse symbiotic partners can also be explained by the studies of Mårtensson *et al.* (1989) that show that legumes can't predict the nitrogen fixation efficiency before nodules are established and fixation is on progress; if so, we may hypothesized that *A. longifolia* emits signals that can be received by several soil bacteria capable of; other authors already suggest a similar behavior, Kiers *et al.* (2003), indicating that legumes can control nodulation through oxygenation of nodule microenvironment, leading to bacteria death and nodule senescence, showing that "the host controls the party" as mentioned by Ferguson *et al.* (2019). Putting it all together, besides this "first sign" generalized symbiosis, this seems to be

easily controlled afterwards considering bacteria fixation inefficiency. However, the reason why it happens remains unclear.

It can also be hypothesized that *Bradyrhizobium sp.* is the mandatory symbiont-partner and, once present, enough nitrogen is given to *A. longifolia*, i.e. a process of specialization seems to occur between *A. longifolia* and *Bradyrhizobium spp.*, allowing this great abundance and preference, which can be observed comparing unburnt and burnt similar intraspecific diversity. This great representation of *Bradyrhizobium spp.* can be since different strains of the same rhizobia can differ in their effectiveness (Dwivedi *et al.*, 2015).

Further studies should rely on a genetic approach in order to understand the presence of such diversity of microorganisms and to answer the following questions: what is the role of the other bacteria, if the hypothesis that nitrogen fixation is guaranteed by *Bradyrhizobium spp.* is taken in account? Can these bacteria be involved in other processes that facilitate both nitrogen fixation or other nutritional or even defense pathways? Can these bacteria behave as opportunistic due to the gain of function through HGT?

This last question can be an interesting hypothesis. In mutualistic associations, where symbioses are included, there is inherent a reciprocal gain relationship between involved partners, it can have an evolution process implicit that could have passed through commensalism (with neutral impact in one partner) or even parasitism (or opportunism) that involves a benefit through a loss (Fig. 23). In fact, besides all the drawbacks that can be faced in a new environment, a whole new "world" of opportunities is open. For this reason, bacteria able to respond to *A. longifolia* nitrogen fixation symbiotic trigger signals could pass through different adaptation processes until mutualism is optimized, and *vice versa*. This adaptation process could allow the permanence of the bacteria inside nodules with no function or even with no impact, detected or not. This hypothesis was proposed by Richardson *et al.* (2000) for possible interactions between IAS and another biota. What if it is hypothesized for belowground microbiota, once our study was performed in young plants or "learners"?

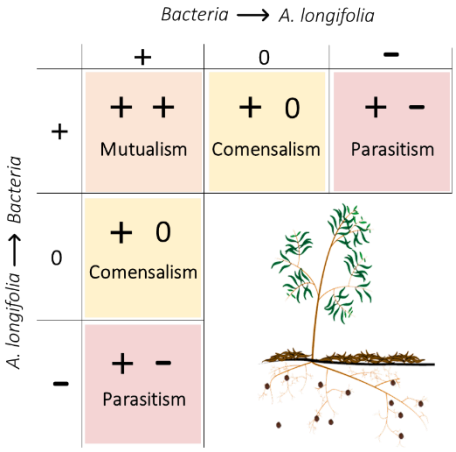


Figure 23 - Possible interactions between *A. longifolia* and bacteria susceptible to its signs adapted from Richardson *et al.* (2000). (+) means a gain, (0) means a neutral effect and (–) means a loss. Squematic representation of symbiotic relation between *A. longifolia* and rhizobia. adapted from Ulm.

In fact, Partida-Martinez and Heil (2011) reported that NRE could interact with hosts in a very complex way and can have mutualistic or antagonistic effects, depending on environmental conditions. A research for more specific genes should be applied in these isolates in order to understand the pathways that they could have a role in (functional approach) and if they are PGPB. In fact, this can be a great hypothesis to take in account once fires may cause the depletion of such a variety of soil components and create a lifeless habitat for *A. longifolia*, making microsymbionts presence priceless.

Another issue that is important to consider is the possibility of symbiotic bacteria can be "buried" in seeds as mentioned by Rodríguez-Echeverría (2010). Further studies should investigate the biogeography of these isolates, in order to explore this hypothesis.

6. Conclusions

A. longifolia is a typical invader, once it can adapt to different conditions like drought, nutrient availability and disturbances. Fire seems to have influence in bacterial diversity inside nodules and its nitrogen fixation functionality; in our study, after this disturbance, *A. longifolia* apparently "selects" nitrogen-fixing bacteria that can be entitled as the "first settlers" within symbiosis reestablishment.

Bradyrhizobium cytisi and other species inside this genus seem to have a determinant role in symbiosis with *A. longifolia*, revealing a close relationship and may have a facilitation effect. However, besides this straight relation *A. longifolia-Bradyrhizobium spp.*, there were reported in our study a considerable bacterial diversity that should be further investigate, especially in what concerns function and biogeography (origin), as well as, signal exchange during infection and nodulation associated with nitrogenase activity.

Ultimately, regarding *A. longifolia* major impacts in its invasive range, a great knowledge of this relation between plant and bacteria could contribute for management and control of this IAS.

7. Future perspectives: where to go?

A. longifolia, as an IAS, is a very interesting plant to study due to its "multi-tasking" behavior ally to surviving in such a dynamic environment. Future perspectives should be constructed in order to (1) study of nodulation in what concerns nitrogenase activity and structural development in time (formation to senescence), since plant germination, under the two conditions study (no fire and fire); (2) the role of water in this process due to the sensibility described for *Acacia sp.* and (3) study of *nif* genes, nod factors and even flavonoids to understand this signal exchange *A.longifolia*-bacteria specificity and get in touch with details of infection process.

The research on *A. longifolia* seeds possible "buried" bacteria can also be interesting because this can make a difference in after fire germination and symbiosis reestablishment.

These future studies should rely on different approaches to provide the most complete knowledge about the subject, going through histological studies, greenhouse essays, isotopic analysis, bacterial functionality and soil analysis and fire intensity implications.

A. longifolia is such an ecosystem transformer and understanding above- and below-ground dynamics is crucial.

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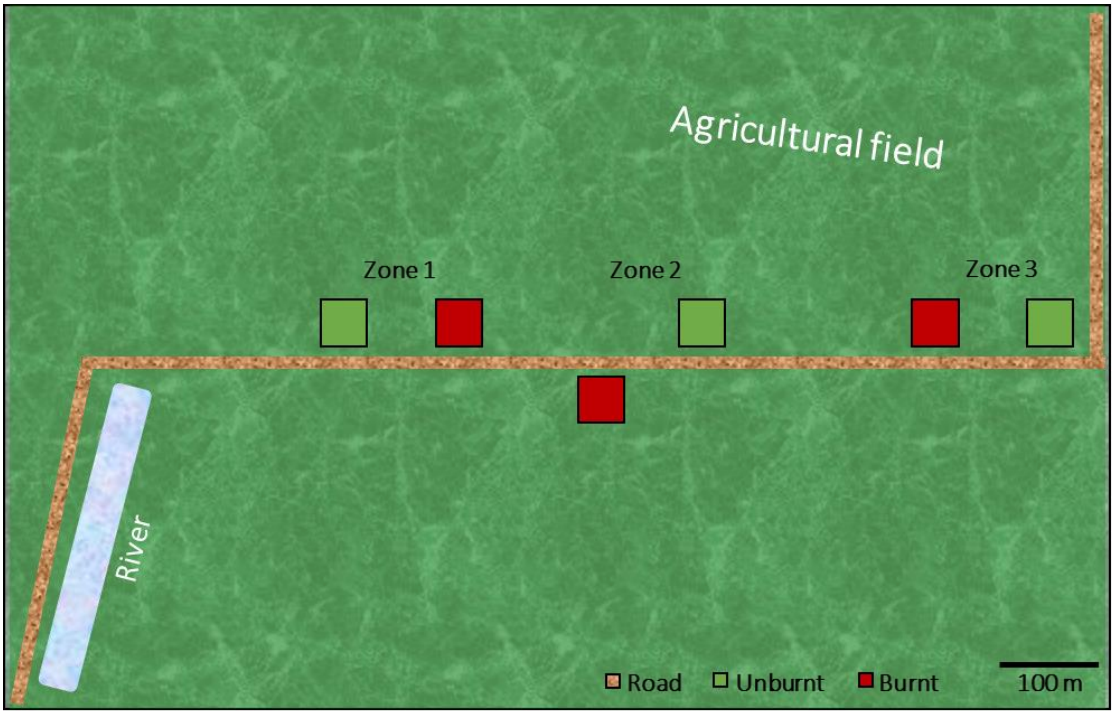
References are done according to Applied and Environmental Microbiology Journal.

Appendix

Appendix 1 – Squematic representation of the location of the six sampled sites: three unburnt zones and three burnt zones (one year after fire).



Appendix 2 – Components of Yeast Mannitol Agar (YMA). Quantities are expressed in grams per liter. The solvent used is distilled water. The pH was between 6.5 and 6.8.

Components	Quantity (g/L)
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Mannitol	5
Yeast Extract	0.4
Agar	20

Appendix 3 - Guanidium thiocyanate, EDTA and Sarkosyl (GES) protocol used for DNA extraction.

DNA extraction protocol using GES (modified)

1. Growth the colonies YMA plates (colonies show be one-day old ideally);
2. Collect one loop (10 µL) to a 2 mL-microtube;
3. Add microspheres (≈ 100 µL);
4. Add 250 µL of lysis buffer (Tris-EDTA-SDS (TES));
5. Incubate 10 min in ice;
6. Vortex for 4 min in maximum speed;
7. Incubate 30 min at 65 ºC;
8. Vortex for 2 min in maximum speed;
9. Add 250 µL of GES reagent;
10. Shake by inversion and incubate for 5-10 min;

    After this step, confirm lysis through the observation of a clear suspension.
11. Add 250 µL of cold ammonium acetate (NH_4Ac 10 M) and incubate 10 min in ice;
12. Add 1 mL of chloroform and isoamyl alcohol (24:1);
13. Mix vigorously by inversion;
14. Centrifuge for 10 min at maximum speed (14000 rpm) and recover the supernatant to a new 1.5 mL- microtube;

    Carefully not to drag the interphase.
15. Add equal volume (1 mL maximum) of cold isopropanol;
16. Mix by inversion;

    Confirm DNA precipitation through the formation of skeins.
17. Centrifuge for 10 min at maximum speed and discard supernatant;
18. Add 1 mL of 70% ethanol to wash the pellet;
19. Repeat step 17;
20. Air dry the pellet during 5-10 min or 15 min at 55 ºC;
21. Solubilize the pellet in 100 µL in 1x Tris-EDTA (TE) buffer.

Appendix 4 - Formulas of Shannon-Wiener diversity, Simpson diversity and Pielou evenness indexes (Krebs, 1989).

- Shannon-Wiener Diversity Index:

$$H' = -\sum_{i=1}^{N} pj\ ln\ pj$$

- Simpson Diversity Index:

$$\boldsymbol{D} = \sum (\boldsymbol{pj} / \boldsymbol{N})^2$$

- Pielou Evenness Index:

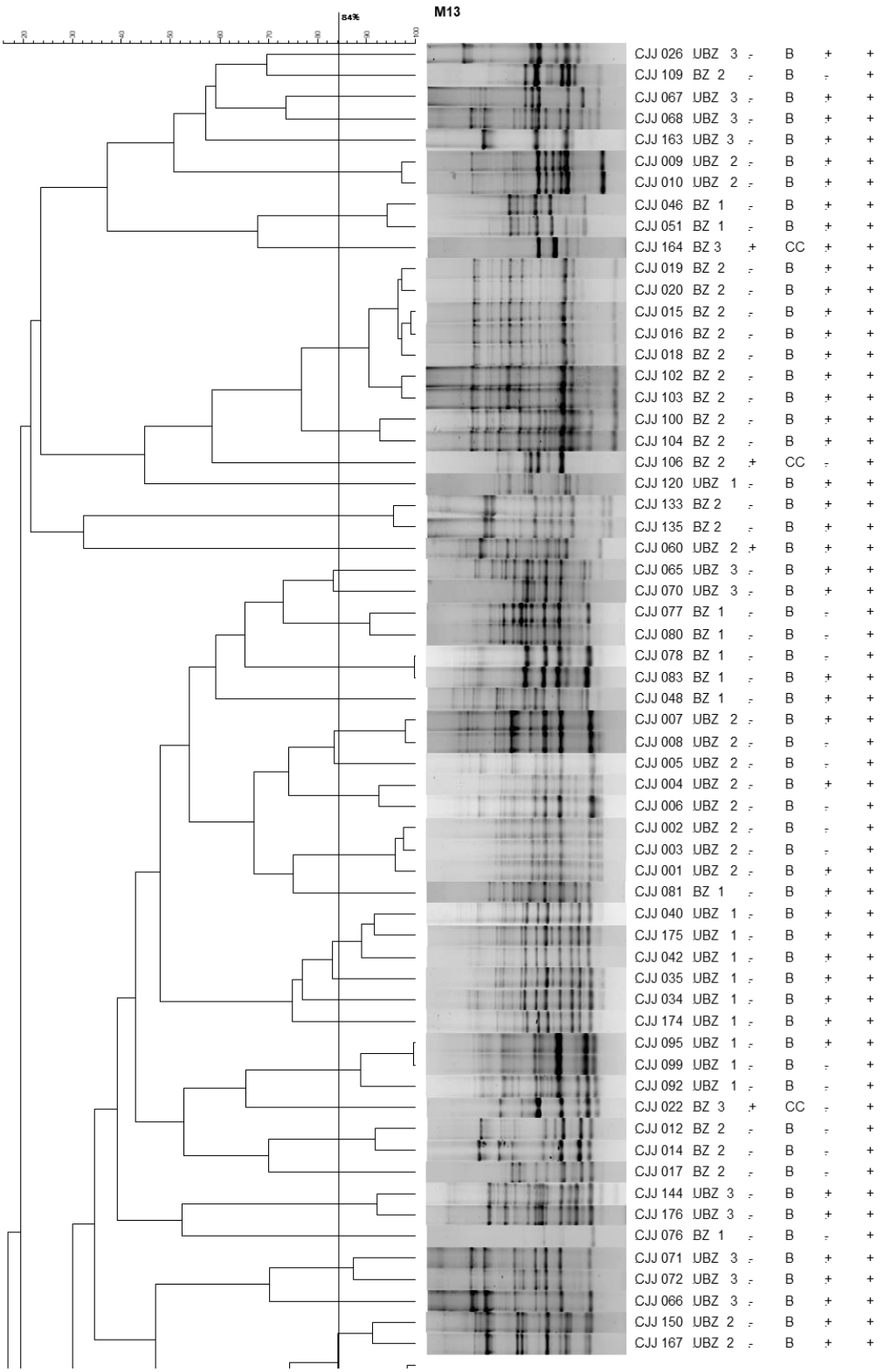
$$J' = H' / H\ max = H' / ln\ N$$

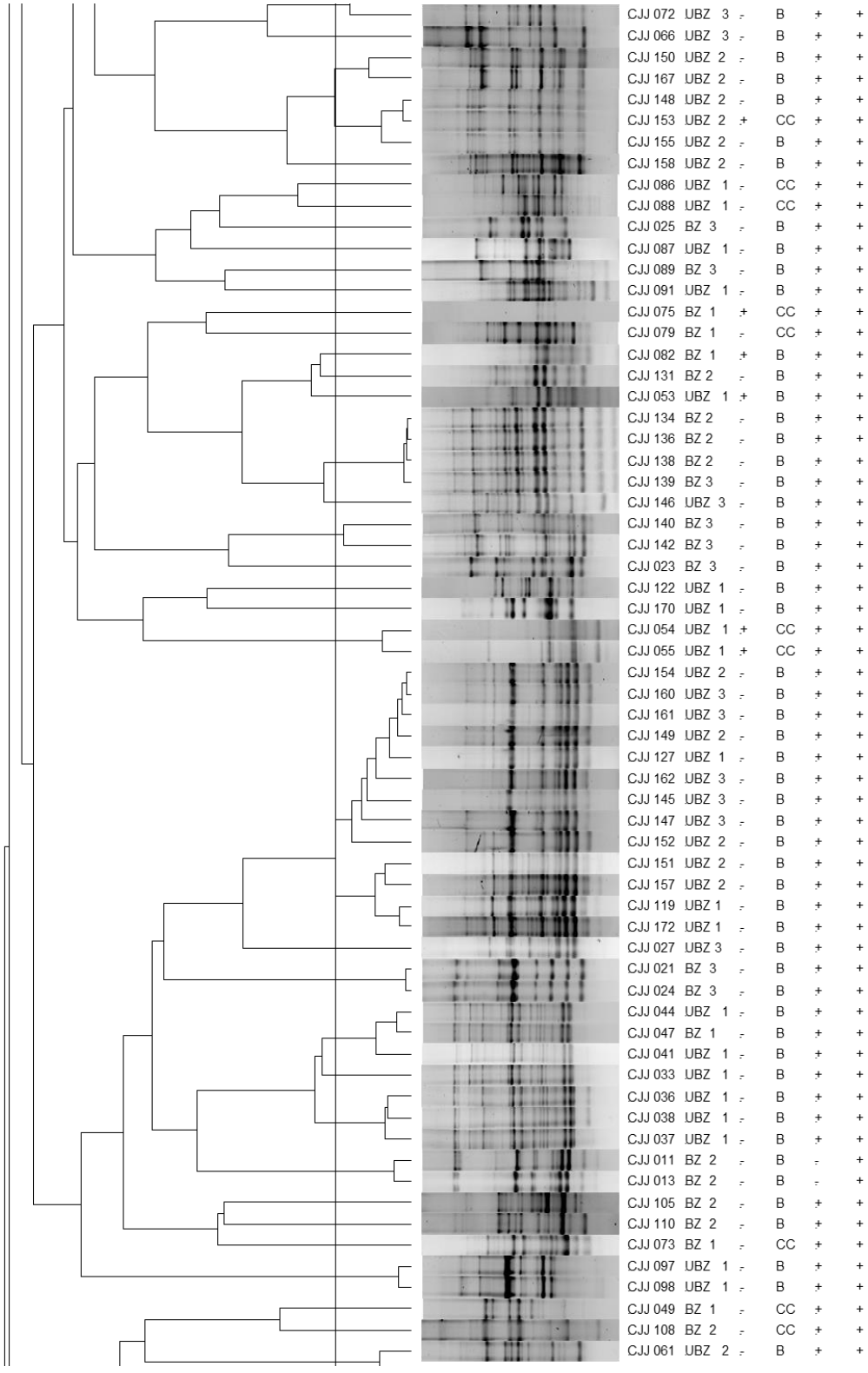
where *pj* is the ratio between the number of strains in each group and the total number, N.

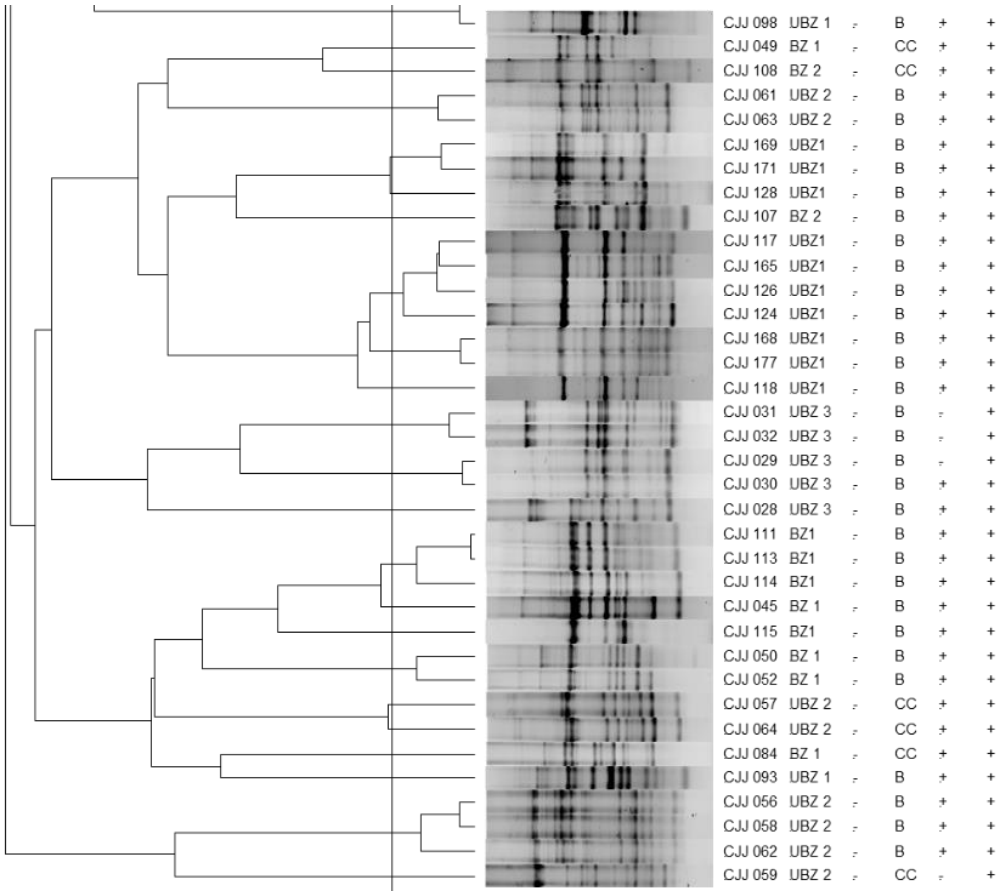
Appendix 5 - Sequences, GC content (%), melting temperature (ºC), length (pb) and product size (pb) of the primers used for 16S amplification.

Primer	Sequence	GC content (%)	Melting temperature (ºC)	Length (pb)	Product size (pb)
PA (8f)	AGAGTTTGATCCTGGCTCAG	50	55	20	≈800
907r	CCGTCAATTCMTTTRAGTTT	30-35	55	20	
104f	AACACATGCAAGTCGAGCGG	55	61.6	20	≈1200
1392r	ACGGGCGGTGTGTRC	50-55	60	15	

Appendix 6 – Dendrogram based on cluster analysis of fingerprinting PCR products amplification, using the unweighted pair-group method with arithmetic mean algorithm (UPGMA) and the Pearson correlation coefficient, of the isolates from nodules of *A. longifolia* young plants. UBZ, isolates from unburnt zones (1, 2 and 3); BZ, isolates from burnt zones (1,2 and 3). 84% was the cut-off used. The figure continues in the two following pages.




CJJ 072 UBZ 3 - B + +
CJJ 066 UBZ 3 - B + +
CJJ 150 UBZ 2 - B + +
CJJ 167 UBZ 2 - B + +
CJJ 148 UBZ 2 - B + +
CJJ 153 UBZ 2 + CC + +
CJJ 155 UBZ 2 - B + +
CJJ 158 UBZ 2 - B + +
CJJ 086 UBZ 1 - CC + +
CJJ 088 UBZ 1 - CC + +
CJJ 025 BZ 3 - B + +
CJJ 087 UBZ 1 - B + +
CJJ 089 BZ 3 - B + +
CJJ 091 UBZ 1 - B + +
CJJ 075 BZ 1 + CC + +
CJJ 079 BZ 1 - CC + +
CJJ 082 BZ 1 + B + +
CJJ 131 BZ 2 - B + +
CJJ 053 UBZ 1 + B + +
CJJ 134 BZ 2 - B + +
CJJ 136 BZ 2 - B + +
CJJ 138 BZ 2 - B + +
CJJ 139 BZ 3 - B + +
CJJ 146 UBZ 3 - B + +
CJJ 140 BZ 3 - B + +
CJJ 142 BZ 3 - B + +
CJJ 023 BZ 3 - B + +
CJJ 122 UBZ 1 - B + +
CJJ 170 UBZ 1 - B + +
CJJ 054 UBZ 1 + CC + +
CJJ 055 UBZ 1 + CC + +
CJJ 154 UBZ 2 - B + +
CJJ 160 UBZ 3 - B + +
CJJ 161 UBZ 3 - B + +
CJJ 149 UBZ 2 - B + +
CJJ 127 UBZ 1 - B + +
CJJ 162 UBZ 3 - B + +
CJJ 145 UBZ 3 - B + +
CJJ 147 UBZ 3 - B + +
CJJ 152 UBZ 2 - B + +
CJJ 151 UBZ 2 - B + +
CJJ 157 UBZ 2 - B + +
CJJ 119 UBZ 1 - B + +
CJJ 172 UBZ 1 - B + +
CJJ 027 UBZ 3 - B + +
CJJ 021 BZ 3 - B + +
CJJ 024 BZ 3 - B + +
CJJ 044 UBZ 1 - B + +
CJJ 047 BZ 1 - B + +
CJJ 041 UBZ 1 - B + +
CJJ 033 UBZ 1 - B + +
CJJ 036 UBZ 1 - B + +
CJJ 038 UBZ 1 - B + +
CJJ 037 UBZ 1 - B + +
CJJ 011 BZ 2 - B - +
CJJ 013 BZ 2 - B - +
CJJ 105 BZ 2 - B + +
CJJ 110 BZ 2 - B + +
CJJ 073 BZ 1 - CC + +
CJJ 097 UBZ 1 - B + +
CJJ 098 UBZ 1 - B + +
CJJ 049 BZ 1 - CC + +
CJJ 108 BZ 2 - CC + +
CJJ 061 UBZ 2 - B + +


CJJ 098 UBZ 1 - B + +
CJJ 049 BZ 1 - CC + +
CJJ 108 BZ 2 - CC + +
CJJ 061 UBZ 2 - B + +
CJJ 063 UBZ 2 - B + +
CJJ 169 UBZ1 - B + +
CJJ 171 UBZ1 - B + +
CJJ 128 UBZ1 - B + +
CJJ 107 BZ 2 - B + +
CJJ 117 UBZ1 - B + +
CJJ 165 UBZ1 - B + +
CJJ 126 UBZ1 - B + +
CJJ 124 UBZ1 - B + +
CJJ 168 UBZ1 - B + +
CJJ 177 UBZ1 - B + +
CJJ 118 UBZ1 - B + +
CJJ 031 UBZ 3 - B - +
CJJ 032 UBZ 3 - B - +
CJJ 029 UBZ 3 - B - +
CJJ 030 UBZ 3 - B + +
CJJ 028 UBZ 3 - B + +
CJJ 111 BZ1 - B + +
CJJ 113 BZ1 - B + +
CJJ 114 BZ1 - B + +
CJJ 045 BZ 1 - B + +
CJJ 115 BZ1 - B + +
CJJ 050 BZ 1 - B + +
CJJ 052 BZ 1 - B + +
CJJ 057 UBZ 2 - CC + +
CJJ 064 UBZ 2 - CC + +
CJJ 084 BZ 1 - CC + +
CJJ 093 UBZ 1 - B + +
CJJ 056 UBZ 2 - B + +
CJJ 058 UBZ 2 - B + +
CJJ 062 UBZ 2 - B + +
CJJ 059 UBZ 2 - CC - +

Appendix 7 - Distribution of nodules with different sizes in roots of an *A. longifolia* young plant.